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Dysfunctional platelet membrane receptors: from humans to mice

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Summary

Insights into hemostasis and thrombosis have historically benefited from the astute diagnosis of human bleeding and thrombotic disorders followed by decades of careful biochemical characterization. This work has set the stage for the development of a number of mouse models of hemostasis and thrombosis generated by gene targeting strategies in the mouse genome. The utility of these models is the in depth analysis that can be performed on the precise molecular interactions that support hemostasis and thrombosis along with efficacy testing of various therapeutic strategies. Already the mouse has proven to be an excellent model of the processes that support hemostasis and thrombosis in the human vasculature. A brief summary of the salient phenotypes from knockout mice missing key platelet receptors is presented, including the glycoprotein (GP) Ib-IX-V and GP IIb/IIIa (α IIb/ β 3) receptors; the collagen receptors, GPVI and α 2 β I; the protease activated receptors (PARs); and the purinergic receptors, P2Y₁ and P2Y₂. A few differences exist between mouse and human platelets and where appropriate those will be highlighted in this review. Concluding remarks focus on the importance of understanding the power and limitations of various in vitro, ex vivo and in vivo models currently being used and the impact of the mouse strain on the described platelet phenotype.

Keywords

Animals; bleeding time; comparative study; disease models; animal; mice; mice knockout; mice mutant strains; tail/blood supply; platelets

Introduction

Gene targeting in the mouse genome is particularly suited for examining the role of platelets in thrombosis and hemostasis. As anucleate fragments of cytoplasm, platelets are refractory to many of the commonly used techniques in cell culture. Thus, the ability to manipulate the mouse genome and subsequently utilize the mouse as a producer of dysfunctional platelets provides a unique in vivo setting to manipulate experimentally the circulating blood platelet. In the knockout of platelet receptors, the mouse models have faithfully mimicked the phenotype of the corresponding human disease if such a human disorder had been described. In other cases, where no known human equivalent has been characterized the mouse models have provided some of the first insights into the physiologic relevance of a specific receptor and ligand interaction. While the utility of mouse models is clear, the inherent differences

between mouse and human platelets and differences between mouse and human hemostasis must be acknowledged and appreciated in the interpretation of data.

Tsakiris et al. have provided a comprehensive overview of mouse hemostasis (1). One of the more obvious differences to human hemostasis is the fact that mouse blood contains a much higher circulating platelet count (2,3). On average, mice have three times more platelets in whole blood as compared to human blood and a fairly wide range of platelet counts among individual mouse strains (2). In addition to the higher platelet count, mouse platelets are smaller than human platelets with a mean platelet volume of 4.7 ± 0.3 fl, as compared to human platelets with a mean platelet volume of 7.5–10 fl (4). The importance of these differences is readily apparent in the inaccuracy of mouse platelet counts obtained in automated cell counters calibrated for human blood. A significant underestimation of mouse platelet count will occur if the automated counter is not specifically calibrated mouse platelets. Manual counts may also be obtained using a hemocytometer and an erythrocyte lysing solution. Together, the higher platelet count and smaller volume probably makes the platelet mass in the mouse blood stream similar between human and mouse blood. The circulating life span of a mouse platelet is also approximately half that of a human platelet and the faster turnover is probably aided by platelet production from megakaryocytes present in the mouse spleen (5). However, the extent of the contribution of mouse spleen-derived proplatelets to the circulating platelet mass is not understood. It is possible that some functional differences could exist between marrow-derived and spleen-derived platelets but no data exists to support such a speculation.

Schmitt et al. (6) have recently provided a summary of the ultrastructural similarities and differences that exist for mouse and human megakaryocytes and platelets. The similarities are much greater than the differences but a few things should be acknowledged. Overall, human and murine platelets have similar compartmentalization within their α -granules and since the platelet release reaction is so important for normal platelet function this should be viewed as evidence that mouse platelets mimic processes that normally occur in human platelets. Besides the size differences mentioned above, Schmitt et al. also report that mouse platelets have an increased granule heterogeneity as compared to their human counterpart. This heterogeneity of granules might be a consequence of the observed membrane partitioning differences between mature human and mouse megakaryocytes (6).

Most of the platelet aggregation agonists that have become common for human platelet studies can be used with mouse platelet-rich plasma or a washed platelet suspension (7). One noticeable exception is ristocetin, commonly used to assay human glycoprotein Ib-IX-V and von Willebrand factor interactions. Ristocetin is unable to agglutinate mouse platelets. Another difference is the efficiency of ADP to stimulate the release reaction from dense granules. ADP can be used as an agonist of mouse platelets but it fails to elicit a maximum platelet aggregation response when used at concentrations comparable to that used with human platelets (7). However, if higher concentrations of ADP are used an irreversible aggregation of mouse platelets can be observed (8,9). In addition, gender differences in the presence of different agonists have been described and should be considered when describing the phenotype from a gene targeting experiment (9,10).

A fairly extensive collection of mice with targeted disruptions in their platelet membrane receptors has been generated in the past several years. While many of the results have been predictable from studies of the inherited human equivalent of the knockout, situations do exist where unexpected findings have been made. For example, one striking difference has been the unexplained alternative use of protease-activated receptors (PARs) as platelet thrombin receptors and this will be described in more detail below. This review focuses on the salient aspects of mouse phenotypes observed in knockout models and, where

appropriate, will highlight differences that exist between the murine and human syndromes. First, we will consider the adhesion receptors, such as those binding von Willebrand factor, fibrinogen and collagen. Then we will consider the thrombin receptors, or PARs, and finish with insights obtained from platelets deficient in adenosine diphosphate (ADP) receptors. Additional membrane receptors contribute to platelet biology but these highlighted receptors represent some of the more obvious candidates for interventional therapy. Indeed, in some instances the described mouse models have become excellent tools to define the importance of a specific receptor and its potential as a target for anti-thrombotic therapies.

The glycoprotein Ib-IX-V complex

The platelet glycoprotein (GP) Ib-IX-V complex is major platelet adhesion receptor encoded by four distinct gene products (11). The major structural and functional subunit of the receptor, GP Ib, is composed of two disulfide-linked subunits each encoded by a separate gene, GP Ib α and GP Ib β . The disulfide bridge linking GP Ib α and GP Ib β is present in the extracytoplasmic portion of each subunit near the single transmembrane spanning region of each polypeptide (Fig. 1). As a separate gene product, the GP IX polypeptide facilitates membrane expression of a GP Ib-IX complex (12). Expression of the GP V gene and polypeptide is not required for the assembly of a GP Ib-IX complex but does assemble with GP Ib-IX and is clearly related to each subunit of the GP Ib-IX as evidenced by the presence of leucine-rich repeats common to all subunits of the GP Ib-IX-V complex (13).

As with most disorders of hemostasis and thrombosis, the clinical manifestation of an absent GP Ib-IX-V complex was characterized long before recognition of the underlying biochemical defect. French physicians, Jean Bernard and Jean Pierre Soulier, originally described the clinical disorder, the Bernard-Soulier syndrome, characterized by mild thrombocytopenia, giant platelets in a blood smear, and absent ristocetin-induced platelet aggregation (14). The molecular basis of the Bernard-Soulier syndrome results from a mutation in either of the 3 genes encoding the GP Ib-IX complex. A model recapitulating the human Bernard-Soulier syndrome was produced by a genetic deletion of the mouse gene for GP Ib α (15). In this case, the mutation reproduced the giant platelet and low circulating platelet count associated with the human syndrome. The characterization of mouse Bernard-Soulier syndrome platelets with ristocetin is not possible as ristocetin-mediated platelet agglutination is species-specific. However, by one criteria of mouse hemostasis, the tail bleeding time assay, the mouse Bernard-Soulier syndrome produces a severe bleeding phenotype (15). Beyond its defect in hemostasis, the mice representing the murine equivalent of the Bernard-Soulier syndrome have no obvious phenotypic anomalies.

The generation of a mouse model of the Bernard-Soulier syndrome also provided “proof of principle” that an absent of the GP Ib-IX complex directly results in the macrothrombocytopenia. In the addition to the abnormal platelets observed on blood smears, the megakaryocytes present in the marrow of Bernard-Soulier mouse display a disordered demarcation membrane system and a reduced cytoplasmic amount of membrane (16). The linkage in producing this phenotype is clearly dependent on the cytoplasmic tail of glycoprotein Ib α as a fusion protein composed of an extracytoplasmic sequence of the interleukin-4 receptor fused to the GPIb α transmembrane and cytoplasmic domains ameliorates the macrothrombocytopenic phenotype (17). Of course, these mice retain their severe bleeding phenotype due to the absence of an amino-terminal domain of GP Ib α and ligand binding sites for von Willebrand factor and thrombin. The same extracytoplasmic region of GP Ib α also interacts with an expanding repertoire of ligands, such as factor XI and Mac-1 (11), and the physiologic relevance of these interactions may also be contributing to some aspects of the bleeding phenotype.

Prior to the development of mouse models of the Bernard-Soulier syndrome a relatively extensive database of mutations associated with human syndrome had been obtained (<http://www.bernard-soulier.org/>). Most mutations are within the gene encoding the GP Ib α subunit while a limited number of mutations have been localized to either the GP Ib β or GP IX genes. The preponderance of mutations in the GP Ib α gene most likely reflects the larger size of the GP Ib α gene and the possible dominance of GP Ib α as the major functional subunit of the receptor. However, in comparing the mouse Bernard-Soulier syndrome to the described phenotypes of individuals diagnosed with the Bernard-Soulier syndrome it is important to consider the heterogeneity of mutations producing the human Bernard-Soulier phenotype. For example, the mouse model of the Bernard-Soulier syndrome was generated by a complete replacement of the all of the nucleotide sequences that encode GP Ib α . In contrast, in the human syndrome many of the mutations produce frame-shifts, or premature stop codons that certainly abrogate surface expression of a GP Ib-IX complex but might allow the partial synthesis of an abnormal subunit. Thus, it would be expected that some subtle differences might exist among individuals with the Bernard-Soulier syndrome and the phenotype found in the murine model.

As an associated subunit of the GP Ib-IX complex, it is worth noting that no human mutations have been found within the GP V that give rise to a Bernard-Soulier phenotype. Indeed, two different genetic knockouts of the mouse GP V gene failed to generate a Bernard-Soulier phenotype (18,19). Instead, the deletion of GP V results in a slightly shortened bleeding time but unable to spread or form thrombi in flowing whole blood (31). The conclusion from such an experiment is that GP VI is not essential as an adhesion receptor but is an important activation receptor. However, a contradictory conclusion was made by Nieswandt and colleagues examining GP VI-deficient platelets generated by antibody (anti-GP VI) removal of the GP VI from the platelet surface (33). In the later study, an examination of thrombus formation was performed in vivo with a damaged carotid artery and a major defect in both adhesion and thrombus formation was noted. Either a genetic or immunological removal of GP VI had little impact on the tail bleeding time in mice.

Comparing the genetic versus immunological removal of GP VI raises questions into how critical is GP VI for platelet adhesion (34). However, a number of differences do exist between the two models. First, is the surprising stability and presence of the FcR- γ subunit in platelets genetically devoid of GP VI. In the antibody-induced depletion of GP VI, a concomitant removal of GP VI and FcR- γ occurs (31). Whether the FcR- γ subunit can assemble with other platelet receptor complexes in the absence of GP VI is still under investigation. In addition, it is unclear what impact the immuno-depletion of a GP VI/FcR- γ complex would have on platelet function mediated by other receptor complexes. As is true in both cases, the conclusions derived from both systems may be biased due to an under appreciation on the net global platelet effect that either genetic and/or immunological depletion causes. Nevertheless, some of the complexity of the platelet response to vascular injury can be appreciated just by acknowledging the similarities that exist between the GP Ib-IX-V receptor and GP VI. Both are critical for the early events of primary hemostasis. GP Ib-IX-V interacts with vWF, whereas GP VI interacts with collagen. Once you move beyond ligand binding both receptors facilitate platelet activation and both must activate a myriad of signal transduction molecules critical for the hemostatic response. The possibility of GP Ib-IX-V/GP VI cross-talk has been recently supported with data characterizing the signaling effects of the snake lectin, alboaggregin A (35). The snake venom protein binds to both GP Ib and GP VI and appears to result in similar signaling events for each receptor (35). A second snake venom protein, Convulxin, was long thought to be a GP VI-specific agonist but has recently been shown to interact with the GP Ib-IX-V complex (36). Whether Convulxin generates platelet activation via the GP Ib-IX-V complex has yet to be explored.

but certainly highlights the potential overlap for receptor responses in the initiation of the hemostatic response.

The platelet protease-activated receptor complexes

Thrombin is a remarkably potent platelet agonist as evidenced by its ability to activate the integrin $\alpha\text{IIb}/\beta 3$, trigger shape change, and release stored activators (37). Beyond its role in platelet activation, it also converts circulating fibrinogen to fibrin monomer. Thus, the contribution and molecular mechanisms by which thrombin signaling contributes to thrombosis is central to an understanding of hemostasis and thrombosis.

Thrombin signals platelets via an elegant mechanism involving protease-activated receptors (PARs) (38). PAR1 became the prototypic member of the family and revealed that thrombin cleavage within the extracytoplasmic portion of PAR1 unmasked a new amino terminus that subsequently could support an intramolecular ligation and elicit transmembrane signaling (39). Such a model is referred to as a tethered ligand where a receptor carries its own silent ligand until the receptor is cleaved, in this case by thrombin. Human platelets can be activated by cleavage of either PAR1 or PAR4 (40). Antibody inhibition of PAR1 blocks platelet activation at low concentrations of thrombin, whereas PAR4 mediates platelet activation only at high thrombin concentrations. Surprisingly, human and mouse platelets differ in their PAR isoforms that they use to mediate thrombin signaling (41). A knockout of mouse PAR1 had no effect on thrombin signaling in mouse platelets (42). In contrast to human platelets, it was later established that mouse platelets express PAR3 and PAR4 (41,43). These data suggested a dual receptor system analogous to human platelets in which the function of human PAR1 was performed by PAR3 in the mouse. However, the transfection of PAR3 into heterologous cells lead to another surprising result when it was observed that PAR3, by itself, could not promote thrombin signaling. However, the cotransfection of mouse PAR3 and PAR4 did result in thrombin signaling at low and high concentrations of thrombin (44). Thus, it appears that mouse PAR3 behaves as a cofactor for cleavage and activation of mouse PAR4, a model that was subsequently proven with the generation of PAR4 deficient mouse platelets (45).

The characterization of the species differences between human and mouse PARs points to the importance of knowing the biological system in question. Although differences exist between mouse and human thrombin signaling it does not preclude the use of mouse models to investigate platelet activation mediated by thrombin. Rather, it points to a situation where the players, in this case the function of a specific PAR, must be defined to understand their role in thrombosis and hemostasis. In both species thrombin is potent platelet activator. Indeed, blocking PAR function or inhibiting thrombin signaling in human platelet should be evaluated as an antithrombotic strategy (46). The utilization of mouse in vitro and in vivo models of thrombosis and hemostasis can provide relevant data on the specific blockade of these important thrombin receptors (47).

Platelet purinergic receptors

Among the receptors described above an overall theme is maintained as each receptor can be crucial during the initial events leading to the development of a thrombus. However, as a consequence of platelet activation ADP is secreted from platelet-dense granules (48,49). In turn, the released ADP can feedback to platelet purinergic receptors, P2Y_1 and P2Y_{12} , as additional agonists of platelet activation. However, the relative contribution of ADP in hemostasis is less than clear but certainly important as demonstrated by the clinical success of P2Y_{12} antagonists. Such antagonists when used at doses that block 40-50% of the P2Y_{12} on the platelet surface have shown efficacy (50).

Platelets from P2Y₁-deficient mice have no apparent problems in their development, survival or reproduction (51,52). However, their platelets are unable to aggregate in the presence of normal concentrations of ADP. Higher concentrations of ADP can induce platelet aggregation but the platelets resist shape change and calcium mobilization. P2Y₁-deficient mice have no spontaneous bleeding but the tail bleeding time is statistically prolonged, as compared to wild-type controls (52). The characterizations of the P2Y₁-deficient platelet provided further support for the presence of a second platelet ADP receptor that was subsequently designated P2Y₁₂. Unlike P2Y₁-deficient platelets, mouse platelets devoid of P2Y₁₂ have a marked prolongation in tail bleeding time assays (8,53). Utilizing P2Y₁₂-deficient platelets Andre et al. (53) have further implicated the P2Y₁₂ receptor in most of the early events of thrombosis, including adhesion. Platelet adhesion on von Willebrand factor was decreased using washed platelets deficient in P2Y₁₂. Thus, the authors suggest an intermediary role for P2Y₁₂ in the signaling steps that lead to α IIb/ β 3 activation and irreversible platelet adhesion dependent upon the two von Willebrand factor receptors, GP Ib-IX-V and α IIb/ β 3. Together, these mouse studies have provided the molecular evidence supporting the clinical efficacy of targeting the P2Y₁₂ receptor with thienopyridine drugs, such as ticlopidine and clopidogrel (54). Indeed, in combination with other platelet antagonists, a future characterization of inhibitory platelet cocktails may provide some exciting possibilities for antithrombotic therapies.

Conclusion

A major consideration for future studies involves the technical issues that surround evaluating mouse hemostasis. A number of methods have described to collect mouse blood (55). Several questions surround the use of mouse blood. Is the platelet pool isolated from a retroorbital plexus equivalent to a platelet pool isolated from a cardiac puncture? In addition, how much influence does the method of collection influence platelet activation during the bleeding procedure? Thus, consideration should be given as to whether a platelet-rich plasma preparation prepared from a venous sinus and collected into a capillary tube is functionally the same as a platelet preparation isolated from terminal procedure, such as a cardiac puncture. A systematic comparison of platelets isolated from each of the vascular beds in the mouse has not been performed.

The tail bleeding time assay has received wide spread use as one indicator of mouse hemostasis. The advantages of the assay are its simplicity and the fact that platelet function is required for the cessation of bleeding. The procedure is not terminal but a second tail bleeding time is not recommended since standardizing the technique to a defined region of the tail is preferred. However, what is the tail bleeding time measuring? There are clearly platelet and coagulation based aspects to the arrest of blood flow in the tail bleeding assay as evidenced by the severe phenotype observed with both models of congenital platelet defects and models of mouse hemophilia (15,56,57). The portion of distal tail removed in different laboratories has not been standardized as some report removing 3, 5 or even 10 mm of distal tail. This region of the tail contains both veins and arteries and depending upon how much of the tail is removed there may be varying degrees of tissue crushing and varying amounts of tissue factor released at the damaged site. Broze et al. have reported a modification of the mouse tail bleeding time assay in which lateral incisions are made to specifically examine vein hemostasis as opposed to arterial hemostasis (58). This type of modification could provide some interesting results for each of the models described above as the various receptor repertoires are evaluated for their contribution to arterial versus venous hemostasis.

Other more elaborate examinations of hemostasis are examining vessels in vivo after injury, such as the circulation in the cremaster muscle, mesentery vessels and carotid arteries. Most commonly, ferric chloride is used to induce damage to the vasculature, but again some

standardization of time of exposure to ferric chloride should be considered since the ferric chloride effects on platelet function may be underestimated. Histological examinations after ferric chloride injury show the injury to be a severe disruption of the endothelial layer (59). In this model it would be important to know which layer of the subendothelium is exposed and is the same layer exposed when different laboratories attempt a similar experiment? Others have chosen to induce thrombi by a small laser injury to the endothelial lining (60). As compared to the ferric chloride model, this probably represents a less severe injury and might lead to results which conflict to that obtained in other injury models. The bottom line in each of these studies is to interpret the results for a given experimental setting and be cautious as to whether any one model is the “best” model of thrombosis. Just as the methods employed to induce thrombosis differ, vascular injury whether it be in a coronary artery or cerebral artery may not elicit an identical physiologic response. In the case of dysfunctional collagen receptors, ex vivo models illustrate an extreme defect in thrombus formation, yet the bleeding time can be essentially normal. Again, interpreting the results as relevant to the specific experimental setting is crucial. Nevertheless, exciting and important results will be obtained as the evaluation of these models of platelet dysfunction continues.

Another potentially unappreciated factor in the interpretation of results is the impact of known, or unknown, pathogens within a specific vivarium. For example, *Helicobacter hepaticus* is a recognized and unwanted pathogen present in many rodent colonies (61). Can pathogens present in one strain, but absent in another, bias published conclusions on the role of a specific receptor or ligand? To date, no information is available, but it is worth acknowledging the studies on another *Helicobacter* species, *Helicobacter pylori*, and a growing awareness for its ability to influence aspects of platelet function (62,63).

A final consideration is the heterogeneity among mouse strains and potential for differences in their response to vascular injury. For example, the thrombin-induced aggregation of platelets from C57B1/6 mice is greater than that of platelets from the 129SvJ strain, the most common lineage for the development of a knockout strain (64). Genetic variation has also been documented in the gene encoding the $\alpha 2$ -subunit of the collagen receptor, $\alpha 2\beta 1$, for the FVB mouse strain (65). A positive side to the heterogeneity of mouse $\alpha 2\beta 1$ levels is a similar documented heterogeneity for human platelets (66,67). Thus, the mouse becomes an interesting model of the physiologic relevance of different collagen receptor levels.

For comparative studies and the generation of an ideal situation, a congenic strain of knockout would be generated and compared to the corresponding wild-type animal of the same strain. A typical breeding scheme to generate a congenic strain requires approximately 10 backcrosses to insure the generation of a congenic colony. In most cases, this requires approximately 2 years of breeding. The time and cost of such a breeding strategy can be high. Thus, the initial characterization of a knockout strain where the control animals represent wild-type littermates from heterozygous (x) heterozygous crosses is to be expected and represents the majority of the experiments that were summarized above. However, each laboratory that has generated a knockout should be attempting the generation of a congenic strain either via traditional methods (10 generations) or via a shortened procedure, such as speed congenics (4-6 generations), offered by Jackson Laboratories (<http://jaxmice.jax.org/services/speedcongenic.html>). The utility of some of these described knockout strains as they are bred into the C57/B16 strain is particularly exciting as a method to exploit the potential for examining the contribution of platelets to atherosclerosis and thrombosis using ApoE (-/-) and LDL receptor (-/-) C57/B16 animals. The utilization of these models may provide insights that bring the biology of these receptors into new areas far beyond their direct involvement in hemostasis and thrombosis. Indeed, this marks a significant direction for future studies that will utilize these models for understanding events relevant to both normal health and the disease state.

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Figure 1. Platelet membrane receptors are depicted that are essential for the initial events of thrombus formation. GP Ib-IX-V, GP IIb/IIIa, GP VI/FcR-γ and α2β1
Each is diagrammatically represented as they might interact with the exposed adhesive ligands within a damaged vascular bed. Subsequent downstream receptors, such as P2Y₁ and P2Y₁₂ are shown responding to ADP released from dense granules following activation. The protease-activated receptors (PARs) are central to the process as thrombin activation receptors. This review highlights the salient features of the phenotype associated with the absence of each receptor focusing on the use of targeted mutations in the mouse germline. The publication (s) describing each knockout is shown for reference.