Published in final edited form as:

Semin Thromb Hemost. 2013 September ; 39(6): 642–655. doi:10.1055/s-0033-1353393.

Glanzmann Thrombasthenia: State of the Art and Future Directions

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Abstract

Glanzmann thrombasthenia (GT) is the principal inherited disease of platelets and the most commonly encountered disorder of an integrin. GT is characterized by spontaneous mucocutaneous bleeding and an exaggerated response to trauma due to platelets that fail to aggregate when stimulated by physiologic agonists. GT is caused by quantitative or qualitative deficiencies of $\alpha\text{IIb}\beta 3$, an integrin coded by the *ITGA2B* and *ITGB3* genes and which by binding fibrinogen and other adhesive proteins joins platelets together in the aggregate. Widespread genotyping has revealed mutations spread across both genes, yet the reason for the extensive variation in both the severity and intensity of bleeding between affected individuals remains poorly understood. Furthermore, while genetic defects of *ITGB3* affect other tissues with $\beta 3$ present as $\alpha v\beta 3$ (the vitronectin receptor), the bleeding phenotype continues to dominate. Here, we look in detail at mutations that affect (i) the β -propeller region of the αIIb head domain and (ii) the membrane proximal disulfide-rich EGF domains of $\beta 3$ and which often result in spontaneous integrin activation. We also examine deep vein thrombosis as an unexpected complication of GT and look at curative procedures for the disease including allogeneic stem cell transfer and the potential for gene therapy.

Keywords

Glanzmann thrombasthenia; $\alpha\text{IIb}\beta 3$ integrin; platelet aggregation; mutation analysis; gene therapy

INTRODUCTION

In Glanzmann thrombasthenia (GT), a rare disease with autosomal recessive inheritance, patients are born with a spontaneous mucocutaneous bleeding syndrome that is variable in

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AUTHORSHIP

ATN and DAW were responsible for manuscript design and writing. XP helped with data collection and Figure design; XP performed the computer modeling. All authors were responsible for approval of the final manuscript. Authors report no conflict of interest.

both frequency and intensity but which on rare occasions is life-threatening.¹⁻³ Excessive trauma-related bleeding is another major problem. Thrombus formation fails in GT as platelets lack or have non-functional α IIB β 3 integrin (originally termed GPIIb-IIIa). GPIIb-IIIa is an essential receptor of platelets that mediates the final step of platelet aggregation induced by physiologic agonists. On platelet activation, the integrin straightens from a bent conformation; in parallel, determinants for the binding of fibrinogen (Fg) and other soluble adhesive proteins are exposed.^{4,5} These proteins mediate aggregation by cross-linking adjacent platelets in a Ca^{2+} -dependent manner.⁶ The α IIB β 3 integrin also transmits the forces that mediate clot contraction and assures the transport of Fg to the α -granules where it constitutes a storage pool. These processes are all modified in GT with variability introduced when mutations allow α IIB β 3 to be at least partially expressed or when function is differentially affected.¹⁻³

In this review we discuss the state of the art with regard to diagnosis and mutation analysis of GT, but start by referring the reader to recent reviews that describe the wide range of mutations that give rise to the disease.^{2,3} To avoid duplication, we will concentrate here on the β -propeller region of α IIB that contains the Ca^{2+} -binding sites and the disulfide-rich EGF-domains of β 3 that are important in determining the activation state of α IIB β 3. We will examine correlations between genotype and phenotype, present deep vein thrombosis as an unexpected complication of GT, assess the use of allogeneic stem cell transplantation as a cure for GT and discuss the current situation with respect to gene therapy.

INITIAL DIAGNOSIS

The diagnosis of GT begins by determining the medical history of the patient. The family history of bleeding and the presence or not of consanguinity should be established and the nature and frequency of bleeding documented. Purpura, petechiae and easy bruising are common features and the disease is mostly, although not always, diagnosed at an early age. Generally in GT the incidence of severe bleeding decreases with age. The platelet count is typically at the low end of the normal range, although cases associated with a moderate thrombocytopenia and platelet size increase have been observed.^{2,3} Platelet function testing rapidly confirms GT for it is the only disorder where platelet aggregation is absent to all agonists (e.g. ADP, collagen, thrombin, arachidonic acid) and while the response to ristocetin is maintained it is sometimes reversible or even cyclic.^{1,7} Clot retraction is mostly absent but can be partial or, for some rare variant forms, normal. Platelets enigmatically fail to spread on collagen. Flow cytometry with a range of monoclonal antibodies (MoAbs) to membrane receptors rapidly confirms the specific deficiency of α IIB β 3.^{1,8} Simple immunofluorescence labeling may suffice if a flow cytometer is not available. For some variant forms, α IIB β 3 is expressed but is not functional; here, diagnosis can be made through the inability of stimulated platelets to bind either PAC-1, a MoAb specific for activated α IIB β 3, or fluorochrome-labeled Fg.^{1,8} As part of a differential diagnosis, chromosome rearrangements associated with leukemia, or the presence of receptor blocking autoantibodies need to be excluded in isolated cases with no family history.⁷⁻⁹ Acquired autoantibodies to α IIB β 3 can on rare occasions block platelet function sufficiently to induce a thrombasthenia-like state.⁹

DETERMINING THE MOLECULAR BASIS FOR THE α IIb β 3 DEFICIENCY IN GT PLATELETS

ITGA2B and *ITGB3* are located at chromosome 17q21.32; but separated at a physical distance of about 3.2 Mbp they are unlikely to share common regulatory domains.¹⁰ α IIb β 3 is at high density on normal platelets with up to 100,000 copies, an expression that varies by at least twofold between individuals.¹¹ Thus, measures of α IIb β 3 copy number need to be supplemented by mutation analysis for identifying carriers of the disease.

A regularly updated database (<http://sinaicentral.mssm.edu/intranet/research/glanzmann>) run by one of us (DAW) lists mutations for 171 patients (May, 2012). Mutations occur across both genes with more affecting *ITGA2B* possibly because, although a smaller gene than *ITGB3*, it has 30 exons compared to 15 (with a doubling of splice sites). Single nucleotide substitutions leading to nonsense or missense mutations, splicing defects and frameshifts, small deletions, insertions and inversions are all common.^{2,3} Missense mutations primarily affect early subunit biosynthesis and/or integrin maturation although some can affect short exonic splicing regulators.^{12,13} Also to be mentioned are amino acid substitutions, primarily affecting β 3, that give rise to alloantigen systems responsible for alloantibody formation and thrombocytopenia following blood transfusion or fetal-maternal incompatibility.¹⁴ In summary, both *ITGA2B* and *ITGB3* are highly polymorphic and prone to mutations.

In GT, most families have their own private mutation although some re-occur in unrelated families and identify gene “hotspots”. GT occurs worldwide but is more abundant in certain ethnic groups (e.g. Iraqi Jews, Palestinian Arabs, French Gypsies of the Manouche tribe) due to the high level of consanguinity within such communities.^{15–17} For example, a common 11-bp out-of-frame deletion (c.2031–2041del, exon 13) in *ITGB3* in the Iraqi-Jewish population of Israel results in a frameshift and premature termination; a 13-bp deletion encompassing the splice acceptor site of exon 4 of *ITGA2B* (IVS3[-3]-418del) is common to Arab kindred in Israel, Saudi Arabia and Jordan; while the French gypsy mutation is a IVS15(+1)G->A substitution that results in a severely truncated α IIb.

Mutation analysis is the key to the complete diagnosis of GT. While sequencing platelet RNA is possible, the small amount and poor stability of RNA in platelets limits this approach and some mutations themselves render the mRNA of the mutated gene unstable.¹⁸ Preliminary PCR-based screening procedures of exons and splice sites such as single strand conformation polymorphism (SSCP) and conformation sensitive gel electrophoresis (CSGE) analyses have been used to pinpoint mutations for sequencing but do not have a guaranteed success. Thus direct sequencing of genomic DNA comprising the 45 exons and the splice sites that compose the *ITGA2B* and *ITGB3* genes is recommended. Mutations must be confirmed by analyzing a second DNA sample; their transmission within families or ethnic groups can be determined using restriction enzyme-based assays or by high-resolution melting point (HRM) analyses.^{1–3,17}

Biogenesis of α IIb β 3 starts in the hematopoietic stem cell (HSC) and continues throughout MK maturation.¹⁹ The α IIb subunit is synthesized with a signal peptide. N-linked glycans are attached to pro- α IIb and to β 3 in the endoplasmic reticulum (ER) where pro- α IIb β 3

complexes form. More carbohydrate modifications accompany the separation of pro- α IIb into mature heavy and light chains in the Golgi apparatus.²⁰ Mutations in GT can block the production of either subunit, interfere with complex formation or interfere with intracellular trafficking.^{20–22} Site-directed mutagenesis involving the co-transfection of the mutated subunit with its wild-type partner in heterologous cells (e.g. transitory expression in COS-7 cells or stable expression in CHO or other cells) establishes the pathogenicity of mutations with recombinant integrin assessed by flow cytometry or by immunoprecipitation followed by SDS-PAGE and immunoblotting (or metabolic radiolabeling).^{20–24} The pro- α IIb and mature α IIb subunits can be distinguished after disulfide reduction.

The expression and functionality of the mutated α IIb β 3 were initial criteria in the classification of GT with patients having <5% residual α IIb β 3 known as type I, 5% to 20% α IIb β 3 as type II and those with substantial to 100% of normal amounts of functionally defective α IIb β 3 as variants.¹ Historically it was assumed that residual α IIb β 3 in type II GT permitted clot retraction and the uptake of Fg into α -granules, a function of α IIb β 3 that involves recycling of the integrin. For example, a type II French patient with a β 3 Cys598Tyr mutation and $\approx$ 10% α IIb β 3 expression had >60% of the normal content of α -granule Fg and platelets that supported clot retraction.^{1,23} Nevertheless, as we will outline, the functionality of the residual α IIb β 3 depends on the modifying effect of the amino acid substitution. If complex formation fails (α IIb with β 3) pro- α IIb undergoes degradation by a calnexin-dependent mechanism.²⁰

GT MUTATIONS MAY HAVE A DIFFERENTIAL EFFECT ON α IIb β 3 AND α v β 3

The first crystal structures of α v β 3 with or without Arg-Gly-Asp (RGD) peptide defined the interactions between the α -subunit β -propeller and the β -I domain of β 3.^{25,26} α IIb and α v propellers share 68% homology and the calcium-binding domains even more. Major structural differences between α IIb and α v β -propellers lie in the upper loops of blades W2 and W3: the 4-1 and 2–3 loops of blades W2 and the 4-1 loop of W3 are domains implicated in ligand binding (see following section).²⁷ Although β 3 associates with α v in normal platelets, only 50 to 100 copies of α v β 3 are surface expressed.⁶ Intriguingly, α v β 3 is primarily associated with intracellular vesicles suggesting its involvement in a transport process.²⁸ Patients with *ITGA2B* mutations blocking α IIb β 3 expression have slightly more α v β 3 in their platelets; but, while mutations preventing β 3 synthesis will delete both α v β 3 and α IIb β 3, some β 3 missense mutations can have a differential effect.^{29–34} For example, a β 3 Leu196Pro mutation yielded platelets with residual α IIb β 3 that underwent a partial clot retraction, yet they could not bind Fg when stimulated.^{29,30} Expression of β 3Pro196 in CHO cells showed a normal biosynthesis and surface expression of α v β 3 whereas α IIb β 3 maturation was retarded.²⁹ However, there was strong inhibition of α v β 3-mediated CHO-cell spreading on immobilized Fg and of fibrin clot retraction, functions that were retained for the poorly expressed α IIb β 3. β 3 Leu262Pro and Ser162Leu mutations also gave platelets with residual α IIb β 3 able to bind fibrin and retract clots but unable to bind soluble fibrinogen when stimulated.^{31,32} The highly conserved Leu262 is in an intrachain loop between Cys232 and Cys273.³⁵ β 3Pro262 transfected in human embryonic kidney (HEK) 293 cells normally formed a complex with exogenous α v and retracted fibrin clots yet the cells failed to interact with immobilized Fg. Structural analysis of α IIb β 3 and α v β 3

revealed that $\alpha v\beta 3$ has several strong interactions that are missing in $\alpha IIb\beta 3$, including the interaction between Trp110 of αIIb with Arg261 of $\beta 3$.³⁵ Overall, $\alpha v\beta 3$ appears more stable than $\alpha IIb\beta 3$ and more tolerant to changes.

Another $\beta 3$ mutation exerting a more deleterious effect on $\alpha IIb\beta 3$ than $\alpha v\beta 3$ is His280Pro (otherwise known as Osaka-5).^{33,34} Whereas platelets from three patients expressed about 6% of normal $\alpha IIb\beta 3$ levels, $\alpha v\beta 3$ was less reduced. Expression of recombinant $\alpha IIb\beta 3$ in HEK293 cells showed that His280Pro allowed a 25% expression of $\alpha IIb\beta 3$ but had no effect on $\alpha v\beta 3$. Previously described Leu117Trp,³⁶ Ser162Leu,³² Arg216Gln,³⁷ Cys374Tyr,³⁸ and a newly created double Arg216Gln/Leu292Ser mutation were also compared with respect to $\alpha IIb\beta 3$ and $\alpha v\beta 3$ expression.³⁴ Whereas all affected $\alpha IIb\beta 3$ maturation, only Leu117Trp and Cys374Tyr $\beta 3$ markedly impaired $\alpha v\beta 3$ expression. Furthermore, it was confirmed that Ser162Leu and Arg216Gln markedly impaired ligand binding to $\alpha IIb\beta 3$ but not to $\alpha v\beta 3$.³⁴ Pulse-chase experiments showed that pro- αIIb Pro280 normally associated with $\beta 3$, yet only small amounts were transported to the Golgi apparatus for cleavage into mature αIIb . In contrast, the processing of pro- αv Pro280 was as for wild-type integrin.

Finally, substitutions within four integrin unique disulfides in EGF domains of $\beta 3$ also affected $\alpha IIb\beta 3$ and $\alpha v\beta 3$ in a different manner.³⁹ Involved were Cys437–Cys457 in EGF-1, Cys473–Cys503 in EGF-2, Cys523–Cys544 in EGF-3, and Cys560–Cys583 in EGF-4. Serine was substituted for each or both cysteines and the mutated $\beta 3$ co-transfected in BHK cells with normal αIIb or normal αv . Disulfide disruption by single serine substitutions resulted in variable constitutive activation of both $\alpha IIb\beta 3$ and $\alpha v\beta 3$ (see later section) with higher integrin expression than after mutation of the many conserved disulfides in this region, suggesting that they do not play a primary structural role in EGF folding. Different effects were noted on the two receptors; for example, disrupting Cys473–Cys503 caused less expression of $\alpha v\beta 3$ relative to $\alpha IIb\beta 3$ whereas disruption of Cys437–Cys457 by Cys457S resulted in a lower expression of $\alpha IIb\beta 3$.³⁹

THE β -PROPELLER ECTODOMAIN OF αIIb IS MUTATION-RICH AND A FREQUENT SOURCE OF GT

The αIIb β -propeller domain is a preferential localization for missense mutations that induce protein misfolding and/or impair calcium binding thereby severely interfering with $\alpha IIb\beta 3$ biogenesis. In 2005, Nelson et al.⁴⁰ reviewed published mutations affecting the β propeller of αIIb highlighting those that disrupt biogenesis by preventing pro- $\alpha IIb\beta 3$ transport from the ER to the Golgi. They also described new Gly128Ser, Ser287Leu and Gly357Ser mutations within blades 2, 5 and 6. Transfection in HEK293 cells showed that although pro- $\alpha IIb\beta 3$ was synthesized in each case, only Gly128Ser totally prevented $\alpha IIb\beta 3$ translocation from the ER to the Golgi with more transport and surface expression for Gly357Ser. Interestingly, Gly128 and Ser287 occur at the interface with the β -I domain, while Gly357 is buried deeper within the propeller. Overall, mutations affecting the interface are more likely to affect pro- $\alpha IIb\beta 3$ maturation than complex formation between pro- αIIb and $\beta 3$. Only Phe171Cys, found in members of a Cypriot family whose platelets lacked $\alpha IIb\beta 3$, was said to stop pro- $\alpha IIb\beta 3$ formation.^{22,40} The highly conserved Phe171 is the fourth residue of a tetra-peptide of a cup motif in which the first and fourth residues build the upper and lower

concentric rings holding Arg261 of $\beta 3$ in place. Modeling showed that Cys171 failed to interact with the βA domain of $\beta 3$.

We update the work of Nelson et al.⁴⁰ in Fig. 1 where a large series of mutations are superimposed onto linear and cartoon representations of the β -propeller. Three historically important mutations occur within or near to Ca^{2+} -binding domain 1 (Gly242Asp), in the zone between domains 2 and 3 (Arg327His), and in a highly conserved region that flanks domain 4 (Gly418Asp).^{41–44} Gly418Asp is in a cage structure and, despite being 8 amino acids N-terminal to the fourth Ca^{2+} -binding domain, disrupts calcium binding. Deletion of Val425 and Asp426 in the fourth Ca^{2+} -binding domain allowed pro- $\alpha IIb\beta 3$ to form but the mutated integrin was not transported to the Golgi.⁴⁵ Other Val298Phe and Ile374Thr mutations were speculated to alter electrostatic potential and through the positioning of the new side chains to affect the stability of the pro- $\alpha IIb\beta 3$ interface.⁴⁶ Glu324Lys, also adjacent to a Ca^{2+} -binding site, severely interfered with $\alpha IIb\beta 3$ expression; unusually, it was repeated in nonrelated families over three continents.^{2,3}

Epidemiological studies on GT have underlined the importance of β -propeller domains as a source of GT. Peretz et al.⁴⁷ investigated 40 Indian families and identified 13 mutations in *ITGA2B* and 10 in *ITGB3* of which many were novel. Included were the αIIb β -propeller substitutions Ala108Val and Gly265Arg. Ala108Val disrupted the interface between αIIb and $\beta 3$. Gly265 serves a hinge function at the beginning of the third β -strand in blade 4; its substitution by the bulky and positively charged Arg was predicted to affect the folding of the β -propeller. Kannan et al.⁴⁸ screened 45 more patients in India and identified 31 mutations (22 novel) in 36 of them. Missense mutations affecting the β -propeller included Leu312Pro, Leu183Pro, Arg327Cys and Gly38His. Mostly they gave a type I phenotype although the previously described αIIb Leu214Arg⁴⁹ had residual expression but with ligand-binding defects. In a French study, Jallu et al.¹² used structural modeling to show how Lys253 of $\beta 3$ has a side-chain that protrudes from the β -I domain towards the αIIb pocket and may be a key residue for the interaction between the β -I domain and the αIIb propeller.

Shen et al.⁵⁰ reported a homozygous Pro126His mutation in a Chinese patient that affected blade 2 of the β -propeller. Transfection of CHO-cells showed no mature αIIb but that the pro- $\alpha IIb\beta 3$ complex formed and co-localized to the ER. Pro126 lies close to the Gly128Ser mutation described by Nelson et al.⁴⁰. Heterozygous *ITGA2B* missense mutations leading to Arg327His (see also ref⁴²) and Gly381Arg mutations were described by us in a German woman whose platelets contained 10–15% residual $\alpha IIb\beta 3$.⁵¹ Arg327 and Gly381 are highly conserved and are situated in blades 5 and 7 of the β -propeller; both affect the interface between the αIIb and $\beta 3$ head domains destabilizing the integrin. Significantly, blades 1–3 of the β -propeller are sufficient for association with $\beta 3$ whereas blades 4–7 are not.⁵²

In contrast, an αIIb Tyr143His mutation gave variant GT and $\alpha IIb\beta 3$ unable to bind PAC-1 and other conformation-dependent MoAbs when stimulated.⁵³ Tyr143 is located in the W3 4-1 loop of the β -propeller. The authors compared the effects of the mutation on the expression and function of recombinant $\alpha IIb\beta 3$ in HEK293 cells to the so-called KO mutation (insertion of Arg-Thr between residues 160 and 161) and Asp163Ala located in the

same W3 4-1 loop. Whereas all mutations abolished soluble Fg and PAC-1-binding functions of α IIB β 3; only Tyr143His allowed partial retention of cell adhesion to surface-bound Fg and of clot retraction. The KO mutation locates to the upper face of the β -propeller.⁵⁴ Alanine substitutions show that Asp163 within the Cys146 to Cys167 loop is a critical residue for ligand binding. In this context, Pro145Ala (W3 4-1 loop) and Leu183Pro (W3 2-3 loop) impair both α IIB β 3 expression and ligand binding function.^{48,55} Also interesting is the Thr176Ile mutation located in the W3:1-2 connecting strand of the β -propeller in a German woman whose platelets contained 25% α IIB β 3 but failed to bind soluble Fg when stimulated although able to adhere to immobilized Fg both in platelets or after transfection in CHO cells.⁵⁶ Significantly, these α IIB mutations that preserve at least in part complex formation are located away from the interface between α IIB and β 3.

A recently reported α IIB Asn2Asp mutation in 4 siblings of an Israeli Arab family affects blade 1 of the β -propeller.⁵⁷ Asn2 is exposed on the β -propeller and is highly conserved. There was no surface expression of α IIB β 3 in platelets of the patient or after transfection of the mutated integrin in BHK cells and while pro- α IIB β 3 complex was formed, trafficking was impaired. Molecular dynamic simulations and modeling of α IIB β 3 showed disruption of a H-bond between Asn2 and Leu366 of a calcium-binding domain in blade 6, thereby impairing calcium binding essential for intracellular trafficking of pro- α IIB β 3. Calcium ions are chelated by conserved Asp or Asn residues in the loops of blades 4-7.⁵⁸ Structural visualization of the WT β -propeller showed close contact between the NH₂ group of Asn2 and the backbone carboxyl group of Leu366 (of blade 6); this contact was eliminated in the mutant, and the distance between the residues increased, resulting in a shifting out of blade 6. There was strong evidence for a H-bond between the side chain carboxamide of Asn2 and the backbone oxygen of Leu266. Interestingly, other “change in charge” mutations such as Glu324Lys, Gly242Asp or Gly418Asp discussed above all resulted in undetectable α IIB β 3 at the platelet surface.

Mitchell et al⁵⁹ suggested that pro- α IIB adopted a bent conformation soon after synthesis and that β 3 did likewise after association with its partner. α IIB contains a conserved glycosylation consensus sequence at N15 (N-X-S/T) and a Asn15Gln mutation prevented pro- α IIB maturation.²⁰ Malfolded proteins may be retained in the ER through chaperone (possibly calnexin) protein interactions. Interestingly, there are four other N-glycosylation consensus sequences in α IIB although their importance for α IIB β 3 biogenesis is unproven.

CYSTEINE MUTATIONS IN THE EGF-DOMAINS OF β 3 ARE OFTEN ACTIVATING

The 56-paired cysteines clustered within the integrin epidermal growth factor domains (IEGF) of the disulfide-rich core of β 3 provide structural restraints to β 3 and indeed all β -integrin subunits.^{6,60,61} Figure 2 shows β 3 IEGF Cys residues mutated in GT. In early work, Cys457Tyr and Cys542Arg mutations in the EGF-1 and EGF-3 domains were shown to prevent more than trace α IIB β 3 expression in platelets and give rise to type I GT.⁶² Transfection in COS-7 cells showed that pro- α IIB β 3 formed but failed to mature. Significantly, a homozygous β 3 Cys560Arg mutation in a French male allowing 20% of the normal α IIB β 3 expression in platelets was shown to lock the integrin in a spontaneously

activated state (i) in his circulating platelets, (ii) after the mutated integrin was expressed in CHO cells and (iii) in a novel conditional mouse model.^{63,64} This gain-of-function mutation allowed spontaneous binding of Fg and MoAbs recognizing activation-dependent determinants on $\alpha\text{IIb}\beta 3$. Despite severely defective platelet aggregation, the patient's platelets bound to and spread on a Fg-covered surface. In the murine model, $\beta 3$ -knockout MKs were modified by human $\beta 3$ -lentivirus transduction and transplantation to provide sufficient levels of the Cys560Arg $\beta 3$ in platelets to investigate how the activated $\alpha\text{IIb}\beta 3$ conformation affected hemostasis *in vivo*. Remarkably, only 35% of mice survived six months after transplant; pathological examination revealed enlarged spleens with extramedullary hematopoiesis and increased hemosiderin indicative of hemorrhage. Thus, continuous occupancy of $\alpha\text{IIb}\beta 3$ with Fg leads to hemorrhagic death rather than a thrombotic state.⁶⁴ In this respect, the results recall platelet-type von Willebrand disease where spontaneous binding of VWF to GPIb α leads to a bleeding syndrome rather than thrombosis.⁶⁵

Mor-Cohen et al²⁴ showed that a homozygous $\beta 3$ Cys549Arg mutation in EGF-3 in 6 Jordanian families with severe GT led to low amounts (1–14%) of constitutively active $\alpha\text{IIb}\beta 3$ spontaneously binding PAC-1 in platelets or after transfection in BHK cells. This mutation broke a conserved Cys549–Cys558 bond in IEGF-3 with mutant pro $\alpha\text{IIb}\beta 3$ largely retained in the ER. Other naturally occurring $\beta 3$ Cys mutations giving rise to activated $\alpha\text{IIb}\beta 3$ include Cys374Tyr, Cys506Tyr, Cys560Phe and Cys598Tyr (data reviewed elsewhere⁶¹). One approach to investigate the role of $\beta 3$ Cys residues has been to systematically mutate them to Ser; alternatively, others have transfected heterologous cells with αIIb and wild-type $\beta 3$ or $\beta 3$ with specific single or double Cys substitutions in EGF or β -I domains.^{39,61,66} Although disulfide disruption mostly resulted in $\alpha\text{IIb}\beta 3$ activation, PAC-1 binding was not always accompanied by Fg binding implying differences in the activation state. Interestingly, disrupting the bond between Cys560 (EGF-3) and Cys583 (EGF-4) gave active $\alpha\text{IIb}\beta 3$ only when Cys560 was mutated suggesting that Cys583 plays a unique regulatory role in $\alpha\text{IIb}\beta 3$ activation, possibly through a disulfide exchange-dependent mechanism.³⁹ A NMR model contradicted earlier work by suggesting that the Cys560–Cys583 bond is positioned within the EGF-4 domain although it continues to play a role in controlling the rigidity of the EGF-3 to EGF-4 interface.⁶⁷

Mutations within integrin unique disulfides in EGF domains of $\beta 3$ also affect the structure and function of $\alpha\text{v}\beta 3$.^{39,66} For example, the double Cys437Ser/Cys457Ser and Cys473Ser/Cys503Ser mutations gave constitutively active $\alpha\text{IIb}\beta 3$ and $\alpha\text{v}\beta 3$; however, the Cys523Ser/Cys544Ser double mutation only yielded constitutively active $\alpha\text{IIb}\beta 3$. Molecular dynamics analysis of a Cys->Ser mutated $\beta 3$ fragment composed of the four EGF domains and β -I domain confirmed that for Cys523–Cys544 the mutated $\alpha\text{IIb}\beta 3$ structure was changed considerably and was stable in its new activated conformation whereas the final $\alpha\text{v}\beta 3$ structure was not modified.³⁹ The Cys523Ser/Cys544Ser mutant was proposed to affect the EGF-2/EGF-1 interface with an opening of the knee domain mediating $\alpha\text{IIb}\beta 3$ activation.

Despite such modifications, disruption of a long-range disulfide between residues Cys406 and Cys655 in $\beta 3$ affected neither the expression nor the function of $\alpha\text{IIb}\beta 3$.⁶⁸ Artificially induced mutations in the extracellular domains of $\beta 3$ that give rise to constitutively active

α IIB β 3 and α V β 3 include Arg305Thr in the β -I domain, Thr562Arg in the EGF-4 domain and Glu552Ala in the EGF-3 domain.^{69–71} Finally, a Met124Val substitution in the β -I domain of β 3 was also said to be activating.⁷²

VARIANT GT

Variants are defined as patients with the clinical phenotype of GT but whose platelets express α IIB β 3 (>20%) in amounts that would normally support platelet aggregation.^{1–3} As stated in the Section “Diagnosis”, variants are characterized by the inability of stimulated platelets to bind soluble Fg or antibodies recognizing activation-dependent determinants on α IIB β 3. They are mostly given by single amino acid substitutions with the nature and position of the affected residue defining the residual functional response. As for the alloantigen systems carried by α IIB β 3, most variant forms concern *ITGB3*. Variant forms of GT have been comprehensively assessed in two recent reviews^{2,3} and only essential findings are summarized here.

(i) Mutations of extracellular domains of β 3

First described was a homozygous Asp119Tyr substitution within the β -I domain preventing α IIB β 3 function without affecting expression.⁷³ Yet, a nearby β 3Leu117Trp mutation gave platelets with <10% α IIB β 3, expression studies showing the retention of malformed pro- α IIB β 3 in the ER.³⁶ Also of historical importance are Arg214Gln or Trp substitutions affecting Ca²⁺-binding and integrin stability.^{74,75} The above mutations all prevent platelet aggregation (and Fg binding), Fg uptake into α -granules and clot retraction. They helped identify sites critical for ligand binding. Mutations such as Asp119Tyr and Arg214Gln or Trp that stop ligand binding without affecting α IIB β 3 maturation seem to escape the ER quality control. The mode of action of these and other mutations within the MIDAS, ADMIDAS and SyMBS domains in β 3 was studied by structural modelling using the crystal structures of α IIB β 3 and α V β 3 as templates.^{2–6,76,77} Another interesting mutation is Ser527Phe in β 3 that induced a high affinity α IIB β 3 receptor by preventing adoption of the bent conformation.⁷⁸ In contrast to the above, nearby Met118Arg, Gly221Asp and Lys253Met mutations give rise to type I GT. Met118Arg and Gly221Asp mutations are located inside the β -I domain and should deeply alter its folding through major changes in size and charge of the side chains. A similar explanation is given for the Met124Val β 3 mutation.⁷² Lys253 has a side-chain that protrudes from the β -I domain and much evidence points to its being a key residue in the interaction with the α IIB propeller, an interaction abrogated by Met253. In contrast, β -I allelic variants that are involved in alloimmune thrombocytopenia (Lys137Gln, Thr140Ile, Arg143Gln, Thr195Asn) concern residues that are located at the surface of the β -I domain with their side chains directed to the outside.

(ii) β 3 cytoplasmic domain mutations

A heterozygous Ser752Pro substitution in the β 3 cytoplasmic tail when combined with a non-expressed allele and 50% α IIB β 3 expression abrogated platelet aggregation.⁷⁹ The platelets of this Argentinian man with mild bleeding supported clot retraction and possessed a normal α -granule Fg pool yet his platelets failed to bind Fg when stimulated (reviewed in detail elsewhere³). A black American child whose platelets also failed to aggregate was

compound heterozygote for a non-expressed allele and a cytoplasmic domain stop codon with $\beta 3$ truncated at Arg724 after only 8 membrane proximal amino acids of the 47 that compose the cytoplasmic tail.⁸⁰ These patients provided key information on the elements in the $\beta 3$ cytoplasmic tail that were responsible for both talin and kindlin-3 binding, steps essential for inside-out signaling.^{3,6} CHO cells expressing $\beta 3$ truncated at Arg724 also failed to spread on Fg confirming defective “outside-in” integrin signaling.

(iii) Macrothrombocytopenia in patients with *ITGA2B* and *ITGB3* mutations

While a normal platelet size and count is a characteristic of GT, a small number of cases provide exceptions.^{2,3,81} An Italian boy with mild bleeding whose platelets aggregate minimally to ADP but which give a slow, irreversible aggregation to thrombin; also showed moderate thrombocytopenia and platelet anisocytosis. Although the surface expression of $\alpha \text{IIb}\beta 3$ was about 18%, the internal pool was maintained. A heterozygous Arg995Gln substitution within the GFFKR sequence of the αIIb cytoplasmic tail was associated with a null allele given by a 13bp intronic deletion (c1440-13_1440-1del) within the splice acceptor for exon 15.^{81,82} Expression studies suggested that the Arg995Gln substitution gave a partially activated integrin.⁸² Five patients from 3 Japanese families with heterozygous *ITGA2B* Arg995Trp gave a similar phenotype when associated with a normal allele.⁸³ Their platelets possessed partially activated $\alpha \text{IIb}\beta 3$ and showed reduced platelet aggregation. αIIb Arg995 and $\beta 3$ Asp723 form a salt bridge and breaking this clasp was proposed as a key step in integrin activation although multiple interactions occur between the integrin cytoplasmic domains.⁸⁴ Enigmatically, macrothrombocytopenia without a significant platelet aggregation defect and no bleeding characterized 5 members of a family with a heterozygous Asp723His substitution in $\beta 3$.⁸⁵ This mutation, also partially activating, resulted in RhoA downregulation when transfected in CHO cells that responded with a unique microtubule-dependent “proplatelet-like” formation when plated on Fg.⁸⁶

Moderate to severe mucocutaneous bleeding, mild thrombocytopenia, large platelets with defective aggregation and up-regulated $\alpha \text{IIb}\beta 3$ activity have since been reported in patients with (i) a heterozygous in-frame deletion causing loss of amino acids 647–686 from the $\beta 3$ transmembrane domain and (ii) a heterozygous Leu718Pro mutation in the membrane-proximal region of the $\beta 3$ cytoplasmic domain.^{87,88} Studies on cultured MKs confirmed that proplatelet formation is negatively influenced by constitutively activated $\alpha \text{IIb}\beta 3$ outside-in signaling.⁸⁹ This may occur from altered interactions with matrix proteins or through secondary transmission of activation to other membrane receptors such as $\alpha 2\beta 1$.⁹⁰

(iv) Mutations affecting kindlin-3 and CALDAG-GEFI

Leukocyte adhesion deficiency-III (LAD-III) syndrome is identified not only by severe bleeding but also recurrent infections not seen in classic GT.⁹¹ Here, a signaling defect common to $\beta 1$, $\beta 2$ and $\beta 3$ integrins means no function in response to stimulation even though the integrins themselves are structurally normal. Infections range from bacterial pneumonia and early septicemia to fungal disease. Osteopetrosis in some patients confirms abnormal osteoclast function. Mutations in *FERMT3* (encoding kindlin-3) account for the phenotype; stop codons or splicing defects leading to truncation and/or non-expression of kindlin-3 predominate.^{91–94} Kindlin-3 belongs to a family of proteins that cooperate with

talin in inside-out integrin activation.⁸⁴ Its binding is specifically inhibited by the GT variant $\beta 3$ Ser752Pro (see Section on Variant-type GT). Defective endothelial cell function may contribute to the severe bleeding in this disease. Bleeding is also accentuated by the defective $\alpha 2\beta 1$ interaction with collagen in the initial platelet interaction with the injured vessel wall.⁹³ An initial report suggested that LAD-III disease was given by mutations of the guanine exchange factor CalDAG-GEFI.⁹⁵ Although this proved untrue, exome analysis has recently identified a homozygous mutation in the *RASGRP2* gene, coding for CalDAG-GEFI in three siblings affected by severe bleeding.⁹⁶ A Gly48Trp substitution abrogated ADP-mediated $\alpha \text{IIb}\beta 3$ inside-out signaling and also affected platelet spreading on an adhesive surface. The result was a selective platelet aggregation defect and defective thrombus formation on collagen. Remarkably, the *RASGRP2* mutation has little impact on leukocyte function.

THE BLEEDING RISK, ASSOCIATED CLINICAL MANIFESTATIONS AND THE UNEXPECTED OCCURRENCE OF DEEP VEIN THROMBOSIS (DVT)

More and more evidence suggests the bleeding phenotype in GT to be multifactorial.^{2,3} Patients show considerable variability, even for siblings within the same family, both in the severity and the frequency of bleeding. Genomic single nucleotide polymorphisms (SNPs) may either reduce bleeding by promoting fibrin formation and/or platelet reactivity with the vessel wall or favor hemorrhage by further dampening platelet function. Initial interest surrounded a potential protective effect of thrombophilic mutations such as Factor (F) V Leiden but there is as yet no evidence that they are more frequent in cases of GT showing lower bleeding phenotypes.³ D'Andrea et al proposed that a milder form of GT occurred in the presence of the *ITGA2* C807T SNP associated with a high density of the $\alpha 2\beta 1$ collagen receptor.⁹⁸ While it is logical that the known wide inter-individual variations in platelet $\alpha 2\beta 1$ or GPVI density influence bleeding in GT, the debate remains open on this.³ The application of genome wide association studies to a large GT cohort is the most likely way to identify markers for residual hemostatic activity and/or bleeding risk.⁹⁹

(i) Is DVT a risk factor in GT?

Patients are not protected against atherosclerosis despite the absence of platelet aggregation but so far there is little evidence of arterial thrombosis or heart disease in GT (reviewed elsewhere²). In contrast, there are an increasing number of reports of DVT in GT (Table I). Remarkably, these include the Argentinian man with variant-type GT and an αIIb Ser752Pro cytoplasmic domain substitution that blocked $\alpha \text{IIb}\beta 3$ activation (see Section Variant GT).^{79,100} A report from Holland identified a man with type I GT with FV Leiden who, on 3 occasions, developed DVT in a lower limb.¹⁰¹ Recurrent DVT was also associated with FV Leiden in a male Brazilian GT patient successfully treated with warfarin.¹⁰² Other cases of DVT did not have known thrombotic risk factors although for two patients, thrombosis occurred after the therapeutic use of FVIIa. As shown in Table I, while many of the patients have not been genotyped, DVT can affect classic or variant forms, is independent of age or sex and can occur in patients with mutations of either *ITGA2B* or *ITGB3*. It appears that DVT is a largely unrecognized risk in GT. Perhaps it is significant that a role has been proposed for GPIb and VWF in venous thrombosis while the

ability of GT platelets to bind fibrin is well known.^{106,107} One of our patients, a 55-yr-old woman with classic type I disease and a homozygous *ITGA2B* missense mutation (Glu324Lys) developed DVT after an air flight. She later showed vascular problems characteristic of Raynaud's phenomenon, and finally this year she presented with chest pains suggestive of coronary heart disease. While coronary angiography first showed a stenosis, this was not seen on a second examination when she was diagnosed with coronary spastic angina and Prinzmetal phenomenon.¹⁰⁵ Thus the absence of platelet aggregation with physiologic agonists had not prevented any of the above cardiovascular or vascular diseases.

(ii) Clinical significance of an $\alpha v\beta 3$ deficiency?

$\alpha v\beta 3$ is involved in so many biological processes that a specific phenotype linked to its deficiency would be expected. While a recent review of the literature found ample evidence that transgenic mice lacking $\beta 3$ show (a) enhanced angiogenesis and tumor growth, (b) facilitated atherosclerosis when fed a high lipid diet, (c) increased bone thickening, (d) altered placental function and elevated fetal mortality and (e) abnormal social behavior; such findings have yet to be documented for human GT.^{2,3} The gene encoding αv (*ITGAV*) is found at chromosome 2q32; remarkably, no mutations have been reported to give inherited disease. While deletion of αv is embryonically lethal in mice, the absence of $\beta 3$ is not and so an alternative explanation is that other integrin β -subunits substitute for $\alpha v\beta 3$ in man. Morgan et al¹⁰⁸ using Cre/loxP technology in mice showed that conditional depletion of myeloid $\beta 3$ -integrins (including osteoclasts) but not from platelets resulted in osteopetrosis and increased tumor growth. This strongly underlines how in mice $\beta 3$ -integrins have cell-specific roles. The question remains as to whether additional phenotypes have so far been overlooked in human GT where the bleeding syndrome predominates for both *ITGA2B* and *ITGB3* defects.

NEW PERSPECTIVES

Treatment in GT has little changed over the years.^{1,109} Two new approaches are autologous stem cell transplantation and gene therapy, these will now be discussed.

(i) Autologous stem cell transplantation

There are now multiple reports of allogeneic stem cell transplantation providing a cure for GT mostly in severely affected children. Initially, bone marrow was obtained from asymptomatic siblings heterozygous for the disease and transplantation performed using conventional protocols.^{110–113} The initial transplant in France was unsuccessful but a second attempt led to the appearance of $\alpha IIb\beta 3$ -expressing and functional platelets and a loss of bleeding symptoms.¹¹⁰ After 2 years the patient was well, mild chronic hepatic graft versus host disease rapidly reversed and he was living a normal life 16 years later.¹¹³ In two more early cases marrow transplantation also led to a clinically stable condition with a loss of major hemorrhage during 18 months and platelets expressing significant levels of $\alpha IIb\beta 3$.^{111,112} Bellucci et al¹¹³ also reported bone marrow transplantation for the sister of the original French case. She had experienced very severe bleeding; furthermore, alloantibodies to $\alpha IIb\beta 3$ had developed after transfusion making the patient refractory to further transfusions. Graft versus host disease was a complication but disappeared on

treatment. Some 3 years later, the patient was living a normal life and platelets with 50% of the normal level of $\alpha\text{IIb}\beta 3$. Anti-platelet antibodies were not present.¹¹³ An adult Japanese patient was reported with GT and acute myeloid leukemia (AML).¹¹⁴ His platelets possessed no $\alpha\text{IIb}\beta 3$; genetic analysis showed a homozygous point mutation and a 9-bp deletion with an Asp133Glu substitution and an in-frame deletion of three amino acids (Val134-Ile-Val) from the W2 β -propeller domain of αIIb . The patient underwent induction therapy for AML and under remission successfully received a bone marrow transplant from an HLA-matched but unrelated donor; conditioning was performed under the cover of multiple transfusions. After 3 weeks, bleeding had stopped and his stable condition was maintained for 2 years with his platelets fully expressing $\alpha\text{IIb}\beta 3$. Flood et al¹¹⁵ successfully performed bone marrow transplants on 3 patients one of whom, a 6-yr-old girl, received an HLA-matched graft from an unrelated donor.

Reduced conditioning is less toxic and was used for stem cell transplantation for children with peripheral blood cells from an unrelated donor.¹¹⁶ Both 100% chimaerism and a mixed chimaera were observed and prevented bleeding with durable engraftment. Unrelated donor-cord blood transplantation was used in three further reports although anti-platelet antibodies were a problem that was raised.^{117–119}

(ii) Gene therapy

There has been an exciting evolution in the use of modified HSCs for genetic therapy of hemorrhagic disorders due to molecular defects within platelet proteins. Three essential issues must be addressed to ensure the success of platelet targeted gene therapy: 1) Can HSCs be given sufficient genetic information to induce abnormal MKs to synthesize transgene products leading to the participation of newly formed platelets in normal hemostasis? 2) Is the newly synthesized molecule maintained as a platelet-specific protein at therapeutic levels for a reasonable period of time? 3) Will the newly expressed protein be tolerated by the immune system or become a target for B- and T-cell mediated immunity with premature destruction and clearing of the genetically-altered MKs and platelets? We now outline the development of pre-clinical gene transfer strategies to help address these questions in GT.

Initial studies revealed that a recombinant retrovirus gene transfer vector under the transcriptional control of a fragment of the *ITGA2B* gene promoter confined transgene expression of a luciferase reporter gene to the MK lineage when tested *in vitro* with human transformed cell lines.¹²⁰ This was followed by the successful use of a gene therapy strategy employing MK-targeted transfer of *ITGB3*, which resulted in restored synthesis of a functional $\alpha\text{IIb}\beta 3$ on the surface of tissue-cultured MKs derived from two patients affected with GT.¹²¹ Lineage-targeted *ITGB3* gene expression was then used in $\beta 3^{-/-}$ mice to correct the GT phenotype *in vivo*.¹²² Transplantation of targeted bone marrow from matched littermates was achieved using $\beta 3^{-/-}$ mice conditioned with complete myeloablation with total body irradiation. Functional $\alpha\text{IIb}\beta 3$ appeared at moderate levels ($\approx 10\%$ of normal receptor levels) on the surface of nearly all peripheral blood platelets.¹²² This was sufficient to restore platelet aggregation and clot retraction *in vitro* and to both improve thrombus

formation within the vasculature and diminish bleeding times with correction of the bleeding phenotype for murine GT.

Next, a strategy to improve long-term bleeding in GT patients was developed that utilized mild sub-myeloablative pre-transplant conditioning to provide the minimum number of functional platelets necessary to improve hemostasis and the quality of life with minimal risk from the transplant protocol. A canine model for GT appeared ideally suited to test a clinically-relevant strategy for human gene therapy.¹²³ Dogs affected with GT due to compound heterozygosity within *ITGA2B* preventing α IIb β 3 expression served as recipients of a therapeutic gene; human α IIb-lentivirus was transduced into CD34+ peripheral blood stem cells (PBC) that were transplanted into GT dogs using low dose pre-transplant conditioning and post-transplant *in vivo* drug-selection to increase the percentage of transduced hematopoietic stem cells.¹²⁴ This approach gave $\approx$ 10% functional platelets with $\approx$ 10% of normal α IIb β 3 levels and measurable platelet aggregation and retraction of a fibrin clot. It was observed that the amount of blood lost from a wound site was reduced 50–135%. The bleeding times were dramatically improved to near normal values with clinical improvement of GT sustained at least five years after transplantation.¹²⁴ Although one dog developed an immune response to the α IIb transgene product, the antibody titer was reduced to background levels with the use of IVIgG (Immunex®) indicating that this potential hazardous side-effect of gene transfer can be alleviated. These data therefore confirm the potential feasibility of platelet gene therapy to control bleeding episodes and improve the quality of life for individuals affected with GT even at moderate transduction efficiencies although potential safety hazards linked to the use of such virus-based vectors will require strict evaluation as will the criteria used for patient selection.

Acknowledgments

ATN acknowledges grants from INSERM (ANR-08-GENO-028-03) and from GIS Maladies Rares. DAW is supported by grants: HL-68138 (DAW) from the National Heart Lung and Blood Institute-National Institutes of Health, by an American Heart Association Award (Northland Affiliate) BGIA 0160441Z and GIA 0755827Z, and generous gifts from the Children's Hospital Foundation, MACC Fund, John B. & Judith A. Gardetto and the Glanzmann's Research Foundation. DAW also acknowledges the following additional affiliations; Childrens Research Institute, Children's Hospital of Wisconsin, Milwaukee, WI 53226; MACC Fund Research Center, Milwaukee, WI 53226.

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Figure 1.

A large series of missense mutations are identified within the α IIb N-terminal β -propeller. Panel (A) is a linear representation. In light blue are domains and mutations at the interface with the β 3 headpiece; in blue marine are those affecting the 7 blades; and in deep blue are those away from the interface. Mutations in green lie within the FG-GAP-G motif while that in red-brown is within a Ca^{2+} -binding domain. Asterisks represent the same mutation found in different patients. Panels (B) and (D) are computer-drawn diagrams of the Ca^{2+} -binding motif and the FG-GAP-G motif respectively. In (C) is a computer drawn ribbon diagram of the β -propeller domain with mutations represented as red graphical sticks. Panel (E) is a Table showing the mutation count as a function of their localization. Models were obtained using the PyMol Molecular Graphics System Version 1.3; Schrödinger LLC (www.pymol.org) and the 3vdo pdb file.^{2,3} The mutations are listed on the GT database (<http://sinaicentral.mssm.edu/intranet/research/glanzmann>).

Figure 2.

Panel (A) is a linear representation of the four consecutive IEGF domains (in grades of green) of the $\beta 3$ subunit with each mutation localized. Asterisks represent the same mutation found in different patients. Panel (B) is a computer drawn ribbon diagram of the IEGF region of $\beta 3$. Mutations are represented as red graphical sticks. Models were obtained using the PyMol Molecular Graphics System, version 1.3, Schrödinger, LLC (www.pymol.org) and the 3ije pdb file.

Table I

GT Patients with DVT described in the literature

Patient	Bleeding	Platelet Aggreg	Residual α IIb β 3	Mutation	Ref	Comments
N° 1 – elderly male	Mild	Absent	50%	β 3 – Ser752Pro	100	Severe proximal DVT and pulmonary embolism after long airflight – Treated with LMWH
N° 2 – adult male	GI bleeding	Absent	Absent/low	Not known	101	Recurrent (3X) proximal DVT in same leg. Factor V Leiden. Treated with LMWH
N° 3 – adult male	Mild	Absent	Absent	Not known	102	Recurrent DVT & possible pulmonary embolism. Factor V Leiden. Treated with heparin and warfarin
N° 4 – elderly woman	Severe	Absent	Absent/low	Not known	103	Repeated GI bleeding, antibodies to α IIb β 3, rFVIIa prior to laparotomy. DVT in both legs + pulmonary embolism Treated with heparin then LMWH
N° 5 – 2yr-old girl	Repeated epistaxis	Absent	Much reduced	Not known	104	Proximal DVT after platelet transfusions (femoral catheter) and rFVIIa. No anticoagulation
N° 6 – adult woman	Moderate	Absent	Trace amounts	β 3 – Cys457Tyr	1 (patient 11) 60	Single episode treated with heparin
N° 7 – adult woman	Severe when child	Absent	Absent	α IIb – Glu324Lys	1 (patient 9) 105	Single episode, no anticoagulation. Raynaud's phenomenon + Prinzmetal angina