CLINICAL, BIOCHEMICAL, AND MOLECULAR ASPECTS OF GLANZMANN THROMBASTHENIA IN HORSES

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CLINICAL, BIOCHEMICAL, AND MOLECULAR ASPECTS OF GLANZMANN THROMBASTHENIA IN HORSES

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Peter W. Christopherson, son of Kathleen Gall and Larry Christopherson, was born on December 17, 1974 in Hartford, Wisconsin. He graduated from the University of Wisconsin-Madison with a Bachelor of Sciences degree in Zoology in May 1997 and a Doctor of Veterinary Medicine in the spring of 2001. On August 14, 2001, he began a Clinical Pathology Residency and PhD program at Auburn University's College of Veterinary Medicine and received board certification by the American College of Veterinary Pathologists in September of 2007.

DISSERTATION ABSTRACT

CLINICAL, BIOCHEMICAL, AND MOLECULAR ASPECTS OF GLANZMANN THROMBASTHENIA IN HORSES

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Glanzmann thrombasthenia (GT) is an autosomal recessive bleeding disorder that is caused by a quantitative or qualitative deficiency in platelet membrane glycoprotein complex IIb-IIIa (integrin αIIbβ3), also known as the fibrinogen receptor. Mutations in either of the genes encoding the αIIb or β3 subunit can cause GT. This disease has been documented at the clinical and molecular levels in both human beings and dogs. In our study, two horses were examined that had a history of chronic epistaxis. Horse 1 was a seven-year old Thoroughbred-cross gelding located in England, while horse 2 was a four-year old American Quarter Horse mare located in Auburn, Alabama. Initial findings for both included normal coagulation screening test results, normal platelet numbers, and normal vWF antigen levels. Clot retraction and platelet aggregation responses to ADP,

collagen, platelet activating factor and thrombin were impaired. Flow cytometric studies indicated a reduction in the αIIbβ3 complex on platelet surfaces. Based on these findings, a diagnosis of Glanzmann thrombasthenia was made for both horses.

The next part of this study entailed the determination of normal cDNA sequences encoding the αIIb and β3 subunits in healthy horses and comparing them to human and canine sequences and cDNA from the horses with GT. The cDNA sequence from both GT horses showed a single guanine to cytosine substitution at position 122 in exon 2 of the gene encoding αIIb. This change results in the substitution of a proline for an arginine and likely causes instability in that region of the protein. Analysis of genomic DNA showed that horse 1 was homozygous and horse 2 heterozygous for this mutation. Based on these findings and the mode of inheritance of GT, it was theorized that horse 2 was a compound heterozygote, and that the mutation in the second allele was causing a lack of mRNA synthesis or nonsense-mediated decay.

Sequence of non-coding areas of the αIIb gene in horse 2 was then compared with gene sequence in normal horses. Results showed that the αIIb gene from horse 2 had one allele with a 10-base-pair deletion that included the splice site between exon 11 and intron 11. This change would be predicted to result in lack of splicing between exons 11 and 12 and the appearance of a premature stop codon 50 base pairs downstream, which likely triggers nonsense-mediated decay of the RNA. The dam of horse 2 was heterozygous for this mutation; the sire was heterozygous for the exon 2 mutation. The sire and dam were clinically normal. This study represents the first characterization of cDNA sequences encoding platelet αIIb and β3 in normal horses and the identification of two distinct mutations causing GT in horses.

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CHAPTER I.

LITERATURE REVIEW

Platelet Structure

Platelets are discoid cytoplasmic fragments that originate from mammalian megakaryocytes and in circulation, have an average lifespan of 7 to 12 days that varies amongst species.[1,2] Platelets are primarily involved in hemostasis but also have roles in other physiologic and pathologic processes such as immunity and inflammation. Major components of platelet anatomy include a plasma membrane, surface receptors, cytoskeletal system, open canalicular system, several types of organelles including a dense tubular system, and three main types of granules. The plasma membrane is composed of an asymmetrically arranged phospholipid bilayer with a hydrophobic core that contains abundant amounts of arachidonic acid, which is converted to different eicosanoids in activated platelets.[3] The outer leaflet of the membrane contains mostly cholinephospholipids (phosphatidylcholine and sphingomyelin), with aminophospholipids (phosphatidylserine and phosphatidylethanolamine) found predominantly in the inner leaflet.[4,5] Under normal quiescent conditions, plasma membrane asymmetry is maintained primarily by ATP-dependent lipid transport driven by aminophospholipid translocase, and to a lesser extent, nonspecific floppase. During platelet activation, the activities of aminophospholipid translocase and nonspecific floppase are diminished by the presence of high levels of cytoplasmic ionized calcium,

which also activates lipid scramblase. Lipid scramblase serves to randomize phospholipids, particularly phosphatidylserine, in the plasma membrane bilayer to provide a negative charge for assembly of coagulation proteins on platelet surfaces.[3,5,6]

Platelet membranes have lipid matrix portions that contain areas high in cholesterol and sphingolipid called microdomains, lipid rafts or detergent resistant membranes.[7] These areas can move laterally and are hypothesized to have a role in coordination of transmembrane signaling of integrins during platelet activation and rearrangement of the actin-rich platelet cytoskeleton during clot retraction.[7] Platelet membranes also contain arachidonic acid (AA), the most abundant membrane fatty acid, and phosphatidylinositol (PI), which is normally found in equilibrium with its derivatives phosphatidylinositol-1-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP2). AA and PI play significant roles in platelet activation.[1,3,8]

Platelet protein and glycoprotein receptors are found both within and outside of the lipid microdomains. Two examples of receptors found within the rafts are glycoprotein Ib-IX-V, the receptor for von Willebrand factor,[9] and glycoprotein VI, a collagen receptor coupled with the Fc receptor gamma-chain (FcR γ-chain or FcRγ), that is responsible for initial collagen binding (GPVI-FcRγ).[10,11] Glycoprotein complex IIb-IIIa (integrin αIIbβ3), the most prominent receptor on platelet surfaces, primarily binds fibrinogen and to a lesser extent von Willebrand factor and is found outside lipid microdomains.[12,13] Other membrane integrins found on platelets include the vitronectin receptor (αvβ3), the major collagen adhesion receptor (α2β1), the fibronectin receptor (α5β1) and the laminin receptor (α6β1).[14-17] GPVI-FcRγ is one of several receptors belonging to the immunoglobulin superfamily found on platelet membranes. Others

include: FcγRIIA (CD32), PECAM-1 (CD31), ICAM-2 (CD102) and the thrombospondin receptor (CD47).[18-22]

G protein-coupled receptors (seven transmembrane domain receptors) on platelets are mainly involved in binding various platelet agonists and the recruitment of additional platelets to the site of vascular injury.[23] ADP receptors P2Y1 and P2Y12 are coupled to Gq and Gi proteins respectively.[24-29] Both are required for a full response to ADP.[30] Three types of protease-activated receptors that bind thrombin have been identified on platelets; PAR1, PAR3 and PAR4.[31] Other G protein-coupled receptors are found on platelet surfaces including those binding thromboxane, prostacyclin, serotonin and platelet activating factor.[32-35]

The platelet cytoskeletal system has three main components: a spectrin-based skeleton, microtubules, and a cytoplasmic actin network. The spectrin-based skeleton is associated with the cytoplasmic surface of the plasma membrane and its strands bind to cytoplasmic actin filaments. This binding aids in maintaining the discoid shape of platelets in circulation.[36,37] In the platelet cytoplasm just subjacent to the plasma membrane there is a circumferential band of microtubules also responsible for maintenance of the discoid shape of unactivated platelets.[38] Microtubules are hollow cylindrical structures composed of laterally associated protofilaments composed of α and β tubulin subunits arranged head to tail.[39] During platelet activation, contraction of the microtubules results in centralization of granule contents and shape change.[3,40] The cytoplasmic actin network comprises the most abundant protein in platelets with over 2 million copies per human platelet.[41] Actin filaments bind with spectrin strands via adducing proteins, while filamin subunits unite actin proteins with the GPIbα subunit of

the GPIb-IX-V complex in the plasma membrane to help stabilize the cytoskeleton.[39,42] During platelet activation and cytoskeletal rearrangement, the severing and disruption of actin filaments is mediated by gelsolin, calcium-activated peptide, and cofilin, which is activated via phosphatase activity.[43] Growth of new actin filaments is facilitated by activation of the Arp (Actin-related protein) 2/3 complex, which can be activated by ActA and Wiskott-Aldrich syndrome protein (WASp) family members.[44,45] Myosin II binding to reorganizing actin filaments provides the contractile force needed for granule centralization within platelets and clot retraction.[46]

An open canalicular system (OCS), or surface connected canalicular system, is a complex organization of membrane-lined channels that extend into the platelet cytoplasm and have openings on the platelet surface in some species.[47] The OCS serves as a reservoir for some of the integrin receptors that are found on platelet surfaces. These receptors can be everted during activation thus exposing more receptors to the extracellular environment.[48] Secretory products, such as granule contents, are often expelled through the OCS, while other particles, such as organisms or proteins, can be taken up through the OCS.[49] Bacteria engulfed by platelets can cause activation, but bacterial killing by platelets has not been documented.[50,51] Ruminant and equine platelets lack an OCS.[52]

Platelets contain several types of organelles including mitochondria, rough endoplasmic reticulum, Golgi complexes, polyribosomes, glycogen stores, a dense tubular system (DTS), and several types of secretory granules.[53] The dense tubular system is found near the microtubules but does not communicate directly with the cytoskeleton. It is a remnant of the smooth endoplasmic reticulum in megakaryocytes

and is comprised of membrane-lined channels that do not communicate with the plasma membrane.[54] The DTS is a site of prostaglandin synthesis, with cyclooxygenase and thromboxane synthetase located on its membrane, and calcium storage; both of which are important during platelet activation and function.

Alpha, dense and lysosomal granules secrete their contents into the extracellular environment by fusing with the plasma membrane or the OCS.[55] Alpha granules are 200-500 nm in diameter and have central and peripheral storage compartments.[56,57] Their contents are either derived from megakaryocytes or are taken up by platelets in circulation. Over 250 different proteins were identified within alpha granules by researchers in one study of human platelets.[58] Many of these proteins are receptors, antigens, GTP-binding proteins, growth factors, coagulation proteins and cell-activating proteins.[53,59] With this variety of contents, alpha granule secretion is involved with a number of processes. Additionally, there is evidence that these granules are compartmentalized and can secrete certain proteins in response to activation.[60] Beta-thromboglobulin, platelet factor 4, PDGF, fibronectin, thrombospondin, vWF, P-selectin, GPIb-IX-V, GPIIb-IIIa, PECAM-1, fibrinogen, and coagulation factors V, VII, XI and XIII are examples of hemostasis-related proteins found within alpha granules.[53,61,62]

Dense granules are named from the electron-dense core seen when viewed by transmission electron microscopy. These granules primarily store ATP/ADP, calcium, serotonin, inorganic phosphates and phosopholipids.[53] They also have protein receptors that become expressed in the membrane after granule fusion including GPIb and GPIIb-IIIa.[63] As with alpha granules, dense granules contain proteins involved in a variety of processes other than platelet function[53,64] While the main components of lysosomal

granules are acid hydrolases, they also have membrane proteins that are expressed after granule secretion.[65]

Platelet Function

Platelets are primarily involved in the initial phases of hemostasis at sites of vascular injury. They also have important contributions in coagulation and have been implicated in several pathologic processes such as thrombosis, atherosclerosis, inflammation and neoplasia.[54,66-68] Activated platelets undergo physical and biochemical changes including shape change, adhesion, aggregation and secretion that result in formation of a platelet plug. During platelet activation, increased concentrations of ionized calcium in the platelet cytoplasm activate several key enzymes involved in these processes (phospholipase C β2, phospholipase A2, protein kinase C, cAMP phosphodiesterase). Calcium is also involved in the expression of a procoagulant surface on platelet membranes through activation of lipid scramblase and inhibition of membrane translocase and floppase.[3,5,6]

Platelet shape change results from reorganization of microtubules and actin cytoskeleton, which results in a normally discoid-shaped platelet developing numerous cytoplasmic projections.[54] Platelets then undergo adhesion to subendothelial collagen under high shear primarily through the binding of platelet GPIb-IX-V to collagen-bound vWF.[3,54] This is followed closely by platelet aggregation via fibrinogen crosslinking to αIIbβ3 on platelet surfaces.[54,69] During activation, platelets release their granule contents into the extracellular space via fusion of granules with the OCS and/or plasma membrane.[70,71]

Platelets can be activated by different types of agonists including ADP, collagen, thrombin, and platelet activating factor.[3] Epinephrine and thromboxane are two other agonists that have variable affects on platelet activation depending on species.[72] Agonist-receptor binding initiates all aspects of platelet function and is accomplished through a series of signal transduction events that include inside-out and outside-in signaling. These events cause physical and biochemical changes within the platelet, which result in shape change, adhesion, aggregation, granule secretion and expression of a negatively-charged, procoagulant surface on the extracellular aspect of the platelet plasma membrane.[54]

Inside-out signaling starts with agonist-receptor binding and results in conformational changes in GPIIb-IIIa, which gives it stronger binding affinity for its primary ligand, fibrinogen. Agonists bound to G protein-coupled receptors (ADP, PAF, thrombin) mediate many platelet activation responses through several mechanisms: increasing cytosolic ionized calcium with resultant protein kinase C activation, reshaping of the actin cytoskeleton, and inhibition of cAMP synthesis.

Ligand binding to G protein-coupled receptors and subsequent G protein signaling activates phospholipase C β2 (PLCβ2), along with other signaling molecules and enzymes.[73] PLCβ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) found in the platelet membrane to inositol triphosphate (IP3) and 1,2-diacylglycerol (DAG).[74,75] IP3 causes calcium release from the DTS stores and influx of calcium from the extracellular environment via ATPase transporters in the OCS and plasma membrane.[23,74] DAG activates protein kinase C (PKC) and, along with ionized calcium, activates diacylglycerol guanine nucleotide exchange factor I (CalDAG-GEFI), which enhances

GTP/GDP switching on Rap1b. Activated Rap1b is involved in the conformation change of αIIbβ3 that results in fibrinogen binding and subsequent platelet aggregation. PKC also has a role in the activation of αIIbβ3 and subsequent aggregation.[76] Collagen, thrombin, PAF and thromboxane receptors also activate the IP3-DAG system.

Actin cytoskeletal reorganization occurs secondary to calcium-sensitive and calcium-insensitive pathways leading to activation of myosin light-chain kinase, which results in myosin phosphorylation. Calcium-calmodulin regulates the calcium-sensitive pathway. The calcium-insensitive pathway involves activation of GTP-binding Rho family proteins, which lead to myosin light chain kinase activation, myosin phosphorylation, and platelet shape change.[77-79] cAMP synthesis is decreased by inhibition of adenylate cyclase via a Gi protein signaling pathway.[80] There are numerous other protein intermediates and alternative pathways involved with signal transduction through G protein-coupled receptors.

Collagen receptor GPVI is responsible for initial collagen binding. Signaling through this receptor involves tyrosine phosphorylation of an immunoreceptor tyrosine-based activation motif present in the FcR γ-chain associated with GPVI by Src kinases.[18,81] This results in other Src tyrosine kinases being attracted and activated, followed by further downstream inside-out signaling leading to activation of phospholipase Cγ2, which has activities similar to PLCβ2. DAG and IP3 are formed, which leads to increased cytosolic calcium and conformation changes in the α2β1 collagen receptor and αIIbβ3.

Ligand binding to integrin receptors, which have undergone conformational changes due to inside-out signaling, initiates outside-in signaling that enhances ongoing

processes and initiates others, including granule secretion, fibrin formation and clot retraction. αIIbβ3 is the most extensively studied platelet integrin, and many researchers have examined its role in the outside-in signaling cascade, which will be discussed later.

Another vital pathway in platelet activation is the release of AA from platelet membranes and its conversion to eicosanoid products especially thromboxane A2.[1,54] Stored as a component of phosphatidylinositol and phosphatidylcholine, arachidonic acid is released from phospholipids in the plasma membrane and dense tubular system. This is mediated by the actions of phospholipase A2 (PLA2) after it is phosphorylated during platelet agonist binding and calcium influx into the platelet cytoplasm.[1,3] AA is subsequently converted to prostaglandin G2 by cyclooxygenase, then to prostaglandin H2 by prostaglandin hydroperoxidase, and finally to thromboxane A2 by thromboxane synthetase.[54,82] Thromboxane A2 is a strong stimulator of platelet aggregation and release of dense granule contents.[3,82] Alternatively, the lipoxygenase system can metabolize AA into two eicosanoids, 12-hydroperoxyeicosatetraenoic acid (12-HPETE) and 12-hydroxyeicosatetraenoic acid (12-HETE), which are compounds that are chemotactic to neutrophils and can inhibit platelet function by inhibition of thromboxane synthesis.[3,83]

The major function of platelets is to maintain vascular integrity through the formation of a primary hemostatic plug. This primary plug, made up of platelets bound to fibrinogen, is fortified by initiation of the coagulation system on platelet surfaces, which results in the formation of thrombin. Thrombin cleaves fibrinogen to fibrin, which is crosslinked by factor XIII to provide a more definitive clot. Platelets have other functions and interactions with other cell types and substrates. It has been shown that

platelets are capable of protein synthesis,[84] leukocyte binding[85] and pro-inflammatory cytokine production.[66] They also are involved in the complex pathophysiologic processes of thrombosis, atherosclerosis and tumor metastasis.[54] Many anti-platelet drugs designed for use in hypercoaguable conditions target αIIbβ3, and additional benefits of such therapies are still being studied.[86]

Integrins

Integrins are one of four classes of cellular adhesion molecules (CAMs) organized by structure. Immunoglobulins, selectins and cadherins compose the other classes of CAMs that have roles in cellular processes including development, motility, proliferation, inflammation, wound healing, immunity and hemostasis.[87-91] Integrins are cell surface transmembrane receptors primarily involved in linking a cell's internal, mainly cytoskeletal, contents with protein ligands found on other cell surfaces and within the extracellular matrix.[92] Examples of extracellular matrix ligands include fibrin, laminin, fibronectin and vitronectin.[87] Soluble ligands can also be binding targets, and there is evidence that integrins also can bind small bacterial and viral proteins.[93,94] Integrins are found on a wide variety of cell types and are heterodimers composed of α and β subunits; 18 α and 8 β subunits have been identified that compose at least 24 different integrins in human beings.[95,96]

The α and β subunits are distinct without any detectable homology. In human beings, sequence identity among the different α subunits is about 30%; β subunits, which are more conserved, have approximately 45% identity.[95] Integrin genes are found on various chromosomes, but there is some gene clustering based on the cells that express

them. For instance, leukocyte integrins are localized on human chromosome 16, while endothelial and platelet integrins are found on chromosome 17 (αIIb, β3) and chromosome 2 (α6, α4, and αv).[95] The two subunits are encoded by separate genes, and both are required for integrin expression on the cell surface.[87,88,97]

Both subunits are characterized by having a large extracellular region hundreds of amino acids in length (>940 residues for α, >640 for β), a single pass transmembrane domain (20-30 residues) and a short cytoplasmic tail (fewer than 75 residues).[88,95,98-100] One exception is the β4 subunit, which has a cytoplasmic tail of ~1,000 amino acids.[101] With the resolution of the crystal structures of most of human integrin αvβ3[102,103] and the binding site of αIIbβ3,[104] the extracellular portions of integrins were found to contain multiple domains. Beginning at the N terminal region, the α subunit is composed of seven β-propeller domain repeats, each approximately 60 amino acids, with calcium-binding motifs found in domains 4-7.[105,106] A group of integrins have an I domain (von Willebrand factor A domain) inserted between β-propeller sheets 2 and 3.[105] An I domain is found in α subunits 1, 2, 10, 11, L, M and X.[107] It reportedly contains a metal ion-dependent adhesion site (MIDAS) and is the major site of ligand-binding.[108-110] The α extracellular region C-terminal to the β-propeller domains is composed of thigh, calf-1 and calf-2 domains.[103]

β subunits have a 50 amino acid N-terminal, cysteine-rich PSI domain (named for its sequence homology in plexins, semaphorins, and integrins).[111] An I-like domain, with a metal-binding sequence similar to the MIDAS found in I-domain-containing α subunits, is sandwiched on both sides by a hybrid domain with which it forms two covalent connections.[99,110] The rest of the extracellular portion of β subunits is made-up of four

cysteine-rich repeats that are termed EGF-like domains, along with a cysteine-rich β-tail domain (βTD).[112,113]

Structurally, based on studies involving αvβ3 and αIIbβ3, integrins have a globular headpiece, composed mostly by the α subunit β-propeller domains (with I domain if present) and thigh domain, along with the I-like domain of the β subunit.[99] In an unactivated state, the rest of the protein complex appears to form hinge and stalk regions.[102-104] In integrins that contain them, the I domain appears to be the major ligand-binding site,[108,109] while integrins without an I domain have major ligand binding in the I-like domain and the interfacing portion of the β-propeller domain. Integrins have several binding motifs, the first one identified was Arg-Gly-Asp (RGD), found in seven integrins including αIIbβ3.[95] This sequence is associated with binding adhesive proteins such as fibrinogen and fibronectin. Several other peptide-binding sequences have been identified.[87]

Transmembrane domains of both subunits are arranged as hydrophobically packed α-helices with some additional complexity in the helix of the α subunit.[114] Both cytoplasmic domains appear to have α-helix structures with C-terminal turns.[115,116] The membrane-proximal regions of the α and β cytoplasmic tails are thought to be connected via a salt bridge involving conserved amino acids (Arg in α, Asp in β) and multiple hydrophobic and electrostatic contacts between the subunits that form a “clasp” and keep the receptor in a low-affinity conformation.[117-119]

Integrins function as a connection between the extracellular environment and actin cytoskeleton of cells and platelets. “Inside-out” signals from the actin cytoskeleton alter integrin binding affinity. The receptor also transmits information about the

extracellular environment to the cytoplasm, which results in cytoskeletal rearrangements.[120]

Integrin activation and subsequent increased affinity and/or avidity for ligand binding can be initiated via inside-out signaling triggered by soluble agonists binding to their receptors on cell surfaces and complex signal transduction pathways.[121] Ligand binding itself also can trigger similar structural changes in integrins by increasing their numbers and distribution on cell surfaces.[122,123] Following agonist binding, protein kinase C (PKC), phosphatidylinositol 3-kinase (PI 3-kinase), and Rap1b (second messenger molecules) have been identified to have a role in the complex organization of inside-out signaling.[121,124] These messengers signal proteins associated with the actin cytoskeleton. This signaling results in reorganization of the cytoskeleton and induction integrin conformational changes.[120]

Results of recent research have implicated the interaction of integrin cytoplasmic domains and Talin1 protein (talin) as a key element in intracellular regulation of integrin activation.[120,125-132] Most of these studies have focused on the β3 integrins, specifically αIIbβ3 and αvβ3. Talin is the most studied and likely most important protein that bridges integrins with the actin cytoskeleton.[127,128] PKCα, Rap1A and the Rap1 interacting adapter molecule (RIAM) are involved in talin activation.[133] Other activators, including phosphatidylinositol 4,5-bisphosphate (PIP2), have been documented.[134] Talin is a 270-kDa cytoskeletal protein composed of an N-terminal head or FERM domain (talin-H) and C-terminal rod domain (talin-R).[135] The protein has binding sites for integrins, actin, and various other proteins.[128] Talin-H is composed of three subdomains termed F1-3, and the

F3 domain has particular importance in binding the cytoplasmic tail of β3 through its phosphotyrosine binding-like domain.[136]

Talin is known to have binding sites for both membrane-proximal and distal residues on the β3 subunit.[125] Talin binding is thought to disrupt the association or clasp between α and β subunits, which causes conformational change in the integrin and increased ligand-binding affinity.[130] Results of recent studies have suggested a model of autoinhibition for talin, where a region of talin-R covers the binding site for the membrane-proximal region of the β integrin subunit.[127] Once talin is activated, it undergoes a change in conformation and binding to the β subunit can occur. Other proteins, such as kindlin-2 (Mig-2), integrin binding protein, and calcium binding protein, bind to the cytoplasmic domains of β3 integrins and contribute to integrin activation.[137-140] The involvement of transmembrane domains in integrin activation is another component of transmembrane signaling and is not completely understood. Data from studies have suggested that transmembrane interactions, including alteration in the number and structure of integrin residues found within the plasma membrane that occurs with disruption of integrin cytoplasmic tails ("push-pull" mechanism), may contribute to inside-out signaling[129,141,142] Inside-out signaling induced by a ligand or other extracellular molecule is an alternative pathway to integrin activation. Currently, there is little scientific evidence for this pathway's contribution to integrin activation.[120]

Inside-out signaling leads to dissociation of the cytoplasmic domains of the α and β integrin subunits and changes the extracellular integrin structure causing an increased affinity for ligand binding.[129] Two models have been proposed to predict the structural changes in the extracellular portion of the receptors. Each results in conformation change

in the ligand-binding pocket of the receptor and increased ligand affinity. Both models start with the receptor in a "bent" conformation with the ligand-binding site poorly accessible because the headpiece is facing the plasma membrane and is in contact with the stalk region. The "deadbolt" model proposes that the integrin stays in the "bent" conformation, but movement in the transmembrane domains causes movement in the stalk regions of the receptor with loss of contact between the stalk and headpiece.[143] The alternative model is the "switchblade" model, where the dislodgement of an EGF-like domain from the β stalk causes the headpiece to extend from its bent conformation in a switchblade-like motion.[144] The events of both models result in increased receptor affinity, ligand binding, clustering of receptors through lateral mobility in the plasma membrane, and transmission of outside-in signals to the interior of the cell and cause rearrangement of the actin cytoskeleton.[120,123]

Outside-in signaling is a complex system composed of enzyme systems, protein complexes, signaling molecules and cytoskeletal proteins that results in reorganization of the actin cytoskeleton with formation of focal adhesions and podosomes.[129,145] Rho GTPases control the formation of focal complexes (small integrin clusters) and focal adhesions (larger integrin aggregates associated with actin fibers).[146,147] Actin-binding proteins such as talin, vinculin, filamin and α-actin connect integrin cytoplasmic tails to actin and myosin contractile fibers.[148]

One enzyme system that is prominently involved in initiation of outside-in signaling is the Src family protein tyrosine kinases (SFKs).[120] Src kinases are composed of SH2, SH3 and kinase domains. When activated by integrin conformational changes, Src kinases elaborate signals from G-protein-coupled receptors, growth factor receptors

and integrins. This leads to changes that contribute to many vital functions, including cell migration, spread, and growth.[149,150] Ten different SFKs have been identified, including c-Src, Fyn, c-Yes, Hck and Lck. Often cells have multiple SFKs that are expressed in different areas of the cytoplasm.[149,151]

Upon activation via inside-out signaling, integrins are bound to ligand and form receptor clusters that correspond to focal complexes and focal adhesions on the cytoplasmic side of the plasma membrane. Even before activation SFKs are found constitutively associated with integrin tails.[120] Within the focal complexes and adhesions, focal adhesion kinase (FAK) autophosphorylates a tyrosine residue to provide a binding site for the c-Src homology 2 domain (SH-2) of an SFK.[121,152] The c-Src-FAK complex amplifies integrin signaling by additional tyrosine phosphorylations and incorporation of more signaling peptides into the complex.[121,152] β3 cytoplasmic tails have direct contact with c-Src SH3 domains and are involved in c-Src regulation.[120] SFKs and FAKs are also involved with several enzymes downstream from integrin signaling, including mitogen-activated protein kinases (MAPK), Rho GTPases, and phosphoinositide 3-kinases.[150,153] Phosphorylation of numerous adapter molecules and GTPases are involved in signal transmission to the actin cytoskeleton in platelets.[154-156] Other phosphorylated proteins activated by Src or related kinases are involved in amplification of platelet outside-in signaling. Phospholipase Cγ produces DAG and IP3, which are products needed for complete platelet aggregation.[157] These two proteins, along with ionized calcium, activate PKC. The cytoplasmic tail of the β subunit is phosphorylated, which facilitates binding of both signaling proteins and myosin. These events are vital to clot retraction and stable aggregation in platelets.[158,159]

The αIIbβ3 Integrin

The integrin αIIbβ3 (glycoprotein complex IIb-IIIa) is the fibrinogen receptor, composed of two protein subunits, αIIb and β3, found on platelets. The primary function of αIIbβ3 is binding of fibrinogen, but it can also bind von Willebrand factor, vitronectin, and fibronectin.[160-164] Both subunits must be present for the receptor to be expressed and functional on the surface of platelets.[97] There are approximately 50,000 to 100,000 of these complexes in each platelet with the majority being expressed on the platelet surface.[165-167] This integrin also is found in the platelet OCS and on the inner membrane of alpha granules.[168,169] Upon activation, platelets undergo several processes including shape change, adhesion to the subendothelium at the site of vascular injury, aggregation, and secretion of their granule contents. During platelet activation, dormant fibrinogen receptors are transported to the platelet membrane via granule fusion with the plasma membrane and evagination of the canalicular system.[165,170]

Much of what is known about integrin structure is based on αIIbβ3 and other β3 integrins. αIIbβ3 was one of the first integrins discovered,[171] and to have its gene cloned[172,173] and sequenced.[174] Genes encoding the αIIb and β3 subunits are found on chromosome 17 in human beings.[175,176] The αIIb gene is approximately 17.2 kilobases and has 30 exons (numbered 1-30) ranging in size from 45 to 249 basepairs.[177,178] Exon 1 encodes a signal peptide that is 31 residues in length.[177,178] The 63 kilobase β3 gene contains 14 exons, designated A through N, that vary from 87 to 430 basepairs in length.[179-181] A 26 residue signal peptide is encoded in Exon A.[174] The two subunits are synthesized individually and form a complex while in the rough endoplasmic reticulum.[97]

The complex is transported to the Golgi apparatus, where the αIIb subunit is cleaved into heavy and light chains that remain linked by a disulfide bond.[165,182,183]

The αIIb subunit heavy and light chains are 837 and 137 amino acids in length, respectively. In its entirety, αIIb is composed of β-propeller, thigh, calf 1, calf 2, transmembrane and cytoplasmic domains.[120] The heavy chain appears to contain the β-propeller, thigh, calf 1, and portions of the calf 2 domain, while the light chain has the remaining portion of the calf 2 domain, along with the transmembrane and cytoplasmic domains.[120] Of particular functional importance are four calcium-binding domains in the αIIb heavy chain found within the β-propeller domain, and their occupancy by calcium is critical for structural stability of the receptor and fibrinogen binding.[165] The β3 subunit is 762 amino acids in length[174] and has PSI, hybrid, I-like, EGF (1-4), βTD and cytoplasmic domains.[120] There is an N-terminal cysteine-rich domain within β3 that is bound by disulfide bonds to 5 cysteine-rich repeats within the body of the protein.[120,184] On the platelet membrane, near the amino termini of both subunits, a combination of the αIIb β-propeller domain and β3 I-like domain form the ligand-binding site.[99]

The GP IIb-IIIa complex is important in both inside-out and outside-in transmembrane signaling by platelets (see integrin section) and fibrinogen binding is responsible for platelet aggregation[185,186] and clot retraction.[187] In addition to its primary functions in platelets, αIIbβ3 binds fibrinogen for uptake and storage by alpha granules[188] and has a role in fibrinogen regulation of proplatelet formation.[189] αIIbβ3 has also been shown to directly interact with and possibly regulate the antidepressant-sensitive serotonin transporter (SERT) in people.[190] Recently, other platelet receptors have been found to have additional interactions with αIIbβ3 signaling pathways. FcγRIIa, an IgG

receptor on human platelets, has been implicated in regulating outside-in signaling of αIIbβ3[191] and contributes to platelet activation secondary to *Staphylococcus aureus* binding by bridging its ligand to fibronectin on αIIbβ3.[192] Platelet endothelial adhesion molecule-1 (PECAM-1) functions as an agonist receptor involved in integrin activation, and it is also a regulator of "outside-in" signaling through αIIbβ3.[22,193]

Glanzmann thrombasthenia

Glanzmann thrombasthenia (GT) is an inherited platelet defect that results from mutations involving the genes encoding αIIb and β3. Dr. Eduard Glanzmann identified the first affected individual in 1918. He observed bleeding disorders in several of his patients who had normal platelet counts but abnormal clot retraction tests.[194,195] By the mid 1970s, other clinical and laboratory abnormalities identified to be associated with this disease included prolonged bleeding times, absence of platelet aggregation in response to several agonists, lack of platelet binding of fibrinogen and decreased expression of both receptor subunits on platelet membranes.[195-200] An autosomal recessive mode of inheritance was suspected owing to the presence of unaffected parents of affected individuals and an even distribution of the disease between males and females. Variable bleeding severity among affected patients were noted.[195,201]

Human patients with GT classically have enhanced bleeding from mucocutaneous tissues such as nasal mucosa, gastrointestinal tract, and gingiva.[195] Other bleeding patterns observed are hematuria, hemarthrosis, bruising, and menorrhagia. Patients do not normally bleed spontaneously, but they can bleed after trauma or have prolonged periods of physiologic bleeds that are normally short-lived in healthy patients, as seen

with eruption of permanent teeth or menstruation.[195] Treatment for Glanzmann thrombasthenia usually is supportive with blood or platelet transfusions administered as needed. Multiple platelet transfusions eventually can lead to development of platelet isoantibodies. Often, the best treatment is to avoid traumatic episodes. Patients with prolonged bleeds should be monitored for iron deficiency anemia, which is the most common complication seen with GT.[195]

The first mutation associated with human GT was discovered in 1990.[202] Mutations associated with GT have been described in Japanese, Indian, African American, Chinese, French Gypsy, Iranian, mixed Caucasian and Algerian human populations.[195] The disease also has been diagnosed and described at the molecular level in Otterhound and Great Pyrenees dogs.[203-205] Over 100 mutations associated with Glanzmann thrombasthenia have been described in human patients. Mutations occur in both αIIb and β3 genes, and compound heterozygotes are common. A complete listing of reported human mutations can be found at http://sinaicentral.mssm.edu/intranet/research/glanzmann/menu.

Classification of GT is based on the amount of detectable receptor, degree of clot retraction and the fibrinogen content of platelet alpha-granules.[171,195,198,201] Quantification of IIb-IIIa complexes can be performed by use of antibody-labeling techniques, flow cytometry or immunoblot techniques. There are three types of GT; Type I, Type II and Variant GT.[195,206] Type I GT is the most common form and Type I GT patients have less than 5% of the normal levels of αIIbβ3, an abnormal clot retraction test, low levels of fibrinogen in their alpha-granules and activated platelets that are unable to bind fibrinogen.[195,206] Vitronectin receptors on the membrane of the platelets are

increased in patients with Type I disease when the mutation involves the gene encoding the αIIb subunit. This is believed to occur because of the increased availability of β3 subunits that normally would have been incorporated into αIIbβ3 complexes. Vitronectin receptors are a complex of the αv subunit and the same β3 subunit found in the fibrinogen receptor.[207]

In Type II GT, which accounts for approximately 14% of the reported human cases, individuals have 10-20% of normal levels of glycoprotein IIb-IIIa, abnormal clot retraction, and low levels of fibrinogen binding and platelet aggregation.[195,206] The mutation in Type II GT is usually found in the β3 gene.[208] Variant GT comprises about 8% of human cases and is typified by IIb-IIIa levels quantified at 50-100% of normal, a normal to absent clot retraction and little or no fibrinogen binding. Similar to Type II GT, Variant GT most often results from a mutation in the β3 gene, but it is a qualitative platelet defect rather than the quantitative defects found in Types I and II.[195,206]

In canine breeds, identified mutations have been limited to the αIIb gene. In 2001, Boudreaux, et al identified a single nucleotide change in exon 12 of the αIIb gene in Otterhounds that corresponded with clinical signs and laboratory findings consistent with Type I GT.[204] This change results in substitution of a histidine for an aspartic acid in the third calcium binding domain, which is a highly conserved area of the protein.[204] Type I GT also has been documented in Great Pyrenees caused by a 14-base-pair repeat in exon 13 with defective splicing of the intron between exons 13 and 14 with the appearance of a premature stop codon 42 bases downstream.[203] This part of the gene encodes the fourth calcium-binding domain and the mutation likely disrupts a large

portion of this domain and eliminates several others that results in an unstable or degraded truncated protein.[203]

New treatment strategies have evolved from molecular research on GT. Gene therapy and bone marrow transplantation are two techniques that are being examined as possible therapies for GT. Bone marrow transplants have been performed with success in both human patients and Great Pyrenees dogs, and stem cell transplants and gene therapy are being studied in people.[209-213]

CHAPTER II.

PLATELET DYSFUNCTION (GLANZMANN THROMBASTHENIA) IN HORSES

Abstract

Glanzmann thrombasthenia (GT) is an autosomal recessive bleeding disorder that is caused by a quantitative or qualitative deficiency in the platelet membrane glycoprotein complex IIb-IIIa (integrin αIIbβ3), also known as the fibrinogen receptor. Mutations in either of the genes encoding the αIIb or β3 subunit can cause GT. This disease has been documented at the clinical and molecular levels in both human beings and dogs. In our study, two horses were examined with a history of chronic epistaxis. Horse 1 was a four-year old American Quarter Horse mare located in Auburn, Alabama, while horse 2 was a seven-year old Thoroughbred cross gelding located in Hatfield, Hertfordshire, England. Initial findings for both included normal coagulation screening test results, normal platelet numbers, and normal vWF antigen levels. Clot retraction and platelet aggregation responses to ADP, collagen, platelet activating factor, and thrombin were impaired. Flow cytometric studies indicated a reduction in the αIIbβ3 complex on platelet surfaces. Based on these findings, a diagnosis of Glanzmann thrombasthenia was made for both horses. This report illustrates a possible differential diagnosis for patients with unexplained bleeding, particularly epistaxis.

Introduction

Glanzmann thrombasthenia is a rare, inherited intrinsic platelet defect involving the platelet fibrinogen receptor (glycoprotein complex IIb-IIIa or integrin $\alpha IIb\beta 3$) that is described in both human beings and dogs.[195,214,215] Horses with clinical features consistent with Glanzmann thrombasthenia have been described,[216,217] but not definitively diagnosed. Clinical signs most commonly observed in human patients and dogs with this disease include purpura, epistaxis, and gingival bleeding. Epistaxis was a prominent clinical feature in the 2 equine cases reported here.

A four-year-old American Quarter Horse mare (horse 1) was examined because of a history of chronic, bilateral epistaxis that was more copious from the left nostril. The horse was in good physical condition (451 kg), and vital signs were normal. Mucous membrane color and capillary refill time were unremarkable. There was a mild anemia (hematocrit 30%; reference interval 32-48%) and a normal platelet count (137,000/µL; reference interval 119,000-247,000/µL). No abnormalities were detected in radiographs of the head, but large petechial and ecchymotic hemorrhages in the nasopharynx were seen on endoscopy and were exacerbated by contact with the endoscope. Routine coagulation screening tests that included activated coagulation time, activated partial thromboplastin time, prothrombin time, thrombin time, and fibrin degradation products were normal, as were plasma concentrations of von Willebrand factor (vWF) antigen (166%; reference interval 70 – 180%). The bleeding time, determined with a spring-loaded cassette on the gingival mucosa, was greater than 60 minutes in the affected horse and less than 2 minutes in a control horse.

A seven year-old, 592 kg Thoroughbred cross gelding (horse 2) was examined for intermittent bilateral epistaxis of $1^{1}/_{2}$ years duration. The owner, who had owned the horse for 3 years, stated that the episodes of epistaxis were unrelated to exercise. Results of hematological evaluation of several blood samples taken over the previous 18 months had been unremarkable. Previous investigations by the referring veterinarian included endoscopic examination of the upper respiratory tract and trachea with concurrent trans-endoscopic tracheal wash, thoracic radiography and thoracic ultrasonography. A focal source of hemorrhage had been located in the mucosa of the left ventral meatus. Pinch biopsies from this area of mucosa had been submitted for histological evaluation, and were normal. The site had been reported to bleed copiously for 2 days after the biopsy was taken.

On physical examination the horse was bright and in good body condition. Vital signs were normal. Clinical findings were normal apart from a small amount of dried blood around the left nostril.

Endoscopic appearance of the upper respiratory tract, including the guttural pouches and trachea, and radiography of the head, did not reveal abnormalities. Routine hematological and biochemical tests revealed no abnormalities; in particular the platelet count was within the normal reference interval (119,000/µL; reference interval 100,000-600,000/µL). Activated partial thromboplastin time, prothrombin time, thrombin time, and fibrin degradation products were normal, as was the concentration of von Willebrand factor antigen (91%; reference interval 80-180%). The gingival bleeding time in horse 2 was greater than 24 hours, and bleeding had to be stopped using a cyanoacrylate glue soaked swab.

The clinical and initial diagnostic findings in both horses were suggestive of a platelet function disorder. Clot retraction tests on citrated whole blood and platelet aggregation studies and flow cytometry using platelet rich plasma were performed on both horses.

Materials and Methods

Clot retraction and platelet aggregation tests[165] were performed on both horses and respective controls. Clot retraction tests used blood collected in a 9:1 ratio with 3.8% trisodium citrate. A small amount of the citrated blood (~500 µl) was used to perform a complete blood count to confirm that thrombocytopenia was not interfering with the test and was not a possible cause for abnormal test results. The remaining blood was used to perform clot retraction tests. The clot retraction test was performed for both affected and control horses as follows: 0.5 ml of citrated blood was added to a plastic conical tube containing 4.5 ml of cold saline, and the tube was mixed gently. Two ml of this mixture were dispersed into each of 2 glass tubes, each containing 10 µl of thrombin (1 unit final). These tubes were capped, gently mixed, and placed in a refrigerator for 30 minutes. The tubes were then transferred to a 37°C water bath and the degree of clot retraction (+1 to +4) was recorded at 1 and 2 hours. This test was always run in duplicate, and the same procedure was used for blood from both suspect and control horses. The degree of clot retraction was recorded after 1 and 2 hours on a scale of 1+ (minimal) to 4+ (maximal).

For platelet aggregation and flow cytometric studies for both horses, blood from affected and control horses was collected into 3.8% trisodium citrate at a ratio of 9:1.

Platelet rich plasma (PRP) was prepared by differential centrifugation of the blood as described.[218] Using PRP from affected horses and PRP from normal horses serving as controls, platelet aggregation in response to several agonists was recorded. Platelet aggregation was measured in a dual channel aggregometer equipped with a strip chart recorder. The final concentrations of agonists evaluated included 2 μM platelet activating factor (PAF), 10, 25, and 100 μM adenosine diphosphate (ADP) and 6, 12, and 24 μg/ml collagen.

Flow cytometry was performed to determine the relative number of fibrinogen receptors present on platelets from both affected horses. Platelet rich plasma was isolated as previously described and 10 μl aliquots were added to 100 μl aliquots of buffer.[205] Monoclonal antibody,[a] specific to the fibrinogen receptor glycoprotein complex IIb-IIIa, was added to samples of diluted PRP from the affected horse and a control horse, and incubated for 20 minutes. Secondary antibody labeled with fluorescein isothiocyanate (Goat $F(ab)_2$ fragment anti-mouse immunoglobulin G), was incubated with the samples for 20 minutes in the dark on ice. Secondary antibody was added alone to PRP from both subject and control horse to assess non-specific binding.

Platelets from affected and control horses were prepared for evaluation via electron microscopy by adding 2 μl prostaglandin E1 (1mg/ml) to 2 ml of citrated PRP, followed by a 5 minute incubation at room temperature. One ml of PRP from each sample was put into a microcentrifuge tubes and 0.75 ml of 0.1% glutaraldehyde in White's saline[219] were added, followed by a 15 minute incubation at 37°C. Platelets then were sedimented in a microcentrifuge, the supernatant was removed, and 0.75 ml 3% glutaradehyde in White's saline were added. Platelets were allowed to fix for 1 hour at

room temperature, after which the supernatant was discarded and the platelet pellets washed with White's saline. Platelets were postfixed in 1% osmium tetroxide in White's saline for 1 hour at room temperature and then dehydrated with a series of ethanols. Propylene oxide was used as a transient solvent. Samples were embedded in Epon 812 resin, and ultrathin sections were stained with uranyl acetate and lead citrate. Examination was performed with a Philips 301 electron microscope.

Results

Electron microscopy was performed on platelets isolated from horse 1, horse 2 and a normal horse. Morphology of the platelets from the affected horses was comparable to that of the normal horse at the electron microscopic level (Figure 1). Clot retraction was markedly reduced in affected horse 1 and was recorded as 1+ after 1 and 2 hours of observation compared to 2+ after 1 hour and 3+ after 2 hours in the control horse (Figure 2). Platelet aggregation responses were markedly impaired in response to all agonists tested (Figure 3). Clot retraction and platelet aggregation responses in horse 2 were markedly reduced and comparable to those described in horse 1 (Figures 2 and 3).

The binding of anti CD41/CD61 (αIIbβ3) monoclonal antibody to platelets from both affected horses was markedly reduced compared to normal equine platelets (Figure 4). Both owners were advised to monitor and record periods of hemorrhage. Blood sampling every six months, or after marked bleeding episodes, was advised to evaluate possible anemia.

Discussion

The petechiation and ecchymoses noted in the nasal passages of horse 1, and the dramatically prolonged bleeding times in horses 1 and 2 were suggestive of a primary bleeding disorder[216] or vasculitis. Vasculitis is often characterized by demarcated areas of skin edema and usually associated with other symptoms of malaise not apparent in these cases.[220] There was no evidence of systemic inflammation, which is normally associated with vasculitis, in the results of tests on blood samples taken from both subjects. Inherited clotting factor deficiencies, the most common being deficiency of factor VIII,[221] or acquired factor deficiencies (caused by warfarin toxicity, end-stage liver disease or disseminated intravascular coagulation)[221] are characterized by abnormal coagulation tests, subcutaneous hematomas, and body cavity hemorrhage including hemarthrosis. Petechial hemorrhages and epistaxis are unusual unless there is concurrent thrombocytopenia, platelet dysfunction or vasculitis.[221]

A platelet disorder was considered the most likely cause of the signs displayed by both cases. Platelet disorders can be subdivided into quantitative and qualitative disorders. Thrombocytopenia was ruled out in both cases, as the platelet counts were normal. Von Willebrand disease, an extrinsic platelet function disorder resulting from reduced levels of vWF was ruled out on the basis of normal circulating concentrations of vWF.

Platelets are required for primary hemostasis.[221] Disruption of the vascular endothelium exposes collagen, which in the presence of von Willebrand factor, results in the adherence and activation of platelets. This activation causes a conformational change

in the fibrinogen receptor or integrin $\alpha IIb\beta 3$ enabling this receptor to bind fibrinogen, which binds other platelets. During and after the platelet plug formation a fibrin mesh forms as a result of the assembly of coagulation factors on the surface of activated platelets.[222] Thus the platelet plug provides a scaffold for secondary hemostasis.

The primary presenting complaint in both horse 1 and horse 2 was epistaxis, which is consistent with the majority of human GT cases.[195] The prolonged bleeding times associated with diminished platelet aggregation responses, reduced clot retraction, and a reduction in the number of fibrinogen receptors was consistent with a diagnosis of Glanzmann thrombasthenia.[195,215]

Two undefined thrombasthenias, with similar characteristics to the cases described, have been reported in horses in Australia and Japan.[216,217] We suggest that thrombasthenia resulting from an inadequate population of fibrinogen receptors on the surface of platelets may be more prevalent in the equine population than presently recognized. Additionally, this condition should be considered as a differential in cases presenting with a bleeding diathesis, but which have normal platelet numbers, normal coagulation screening tests, normal concentrations of vWF, and no evidence of vasculitis.

Footnotes
[a]Mouse anti-ruminant CD41/CD61, MCA1095, Serotec, Raleigh, NC

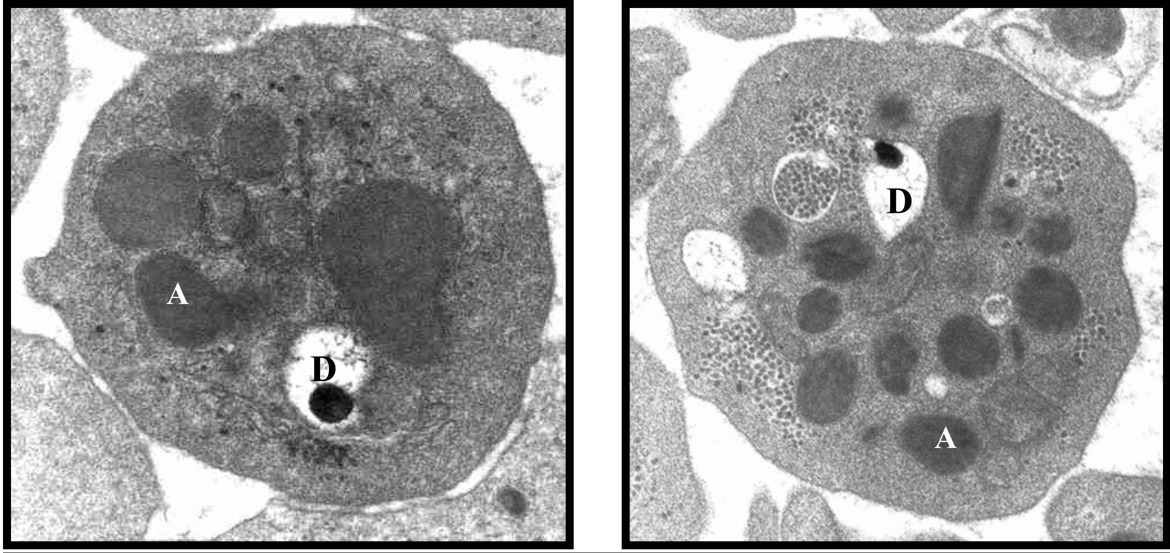


Figure 1. Transmission electron micrographs of platelets from a normal horse (left panel) and a horse with Glanzmann thrombasthenia (horse 1; right panel). Platelets from both horses had normal morphology, including the presence of alpha (labeled A) and dense (D) granules.

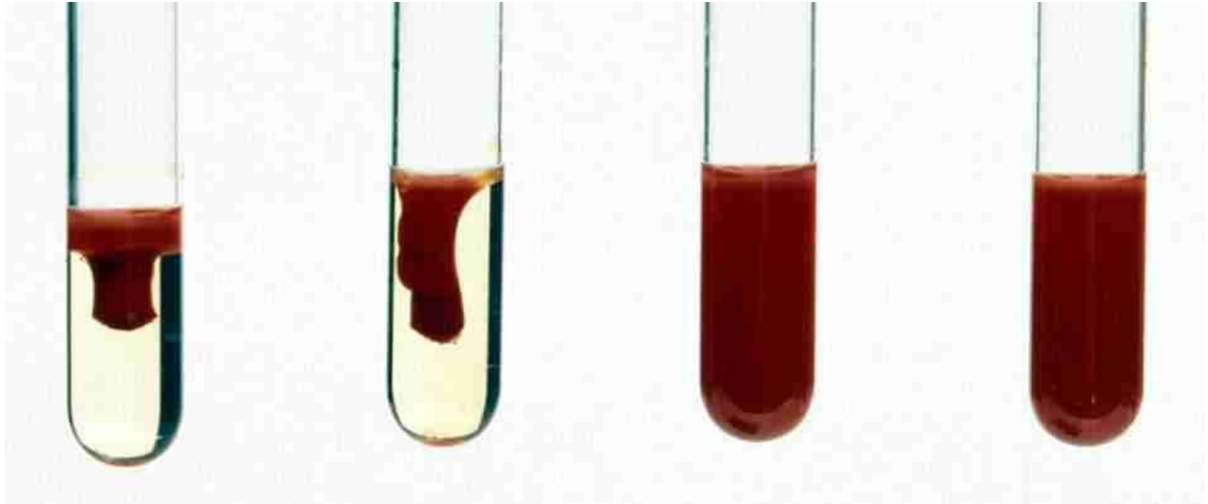


Figure 2. Clot retraction tests from a control horse and a horse with Glanzmann thrombasthenia (horse 1). The two tubes on the left are from the control horse and demonstrate normal (+3) clot retraction after 2 hours. The two tubes on the right contain blood taken from horse 1, and show markedly reduced clot retraction. A similar reduced clot retraction response was observed in horse 2 (not shown).

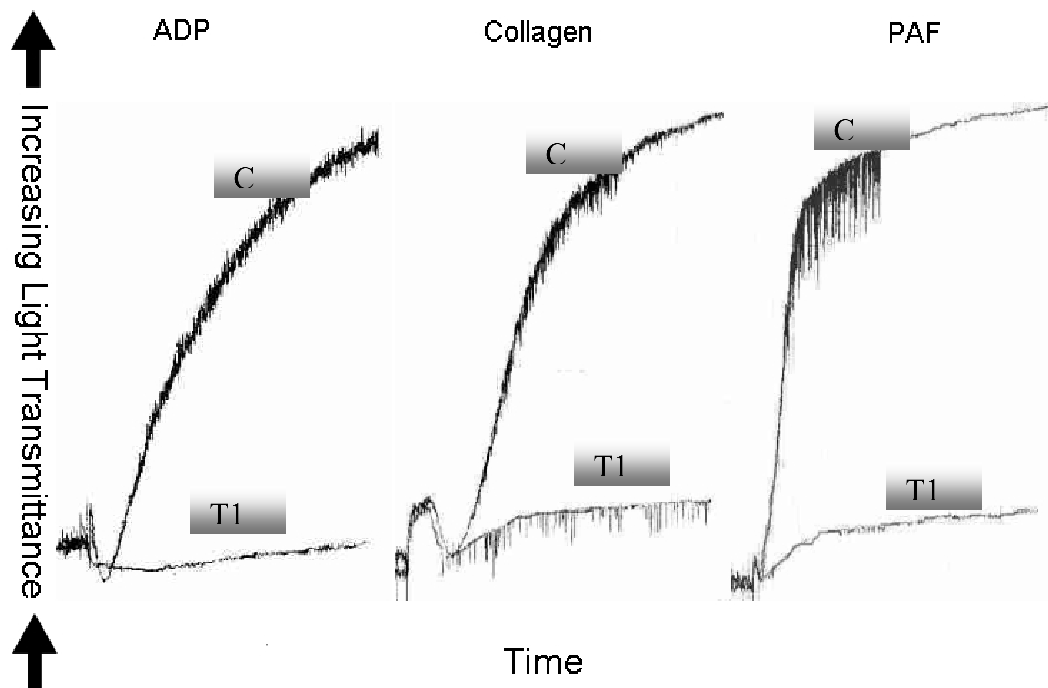


Figure 3. Platelet aggregometry tracings from control horse and a horse with Glanzmann thrombasthenia (horse 1). Traces from the control horse are labeled C, traces from the affected horse are labeled T1. Aggregometry tracings demonstrate markedly reduced platelet aggregation responses to ADP, collagen and PAF in horse 1 (reduced light transmittance) compared to a normal control horse. Similar responses were observed to all concentrations of agonists. Aggregometry responses in horse 2 (not shown) were similar to those observed in horse 1.

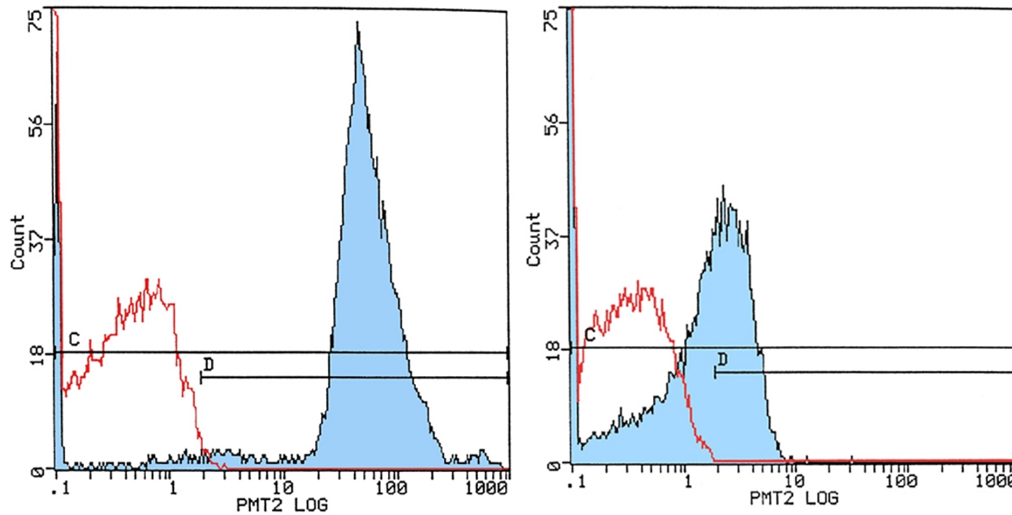


Figure 4. Flow cytometric analysis of the binding of anti-CD41/CD61 (αIIbβ3) antibody to platelets from a control horse and a horse with Glanzmann thrombasthenia (horse 1). The unshaded peaks (to the left of gate D) represent non-specific binding of secondary FITC-labeled antibody only. The shaded peaks (within gate D) represent binding of antibody to control horse platelets (left panel) and affected horse platelets (right panel) incubated with primary and secondary antibody. Binding of antibody to platelets of horse 1 was markedly reduced when compared to binding of antibody to normal horse platelets. Flow cytometric results obtained using platelets from horse 2 (not shown) were comparable to those seen with horse 1.

CHAPTER III.

CHARACTERIZATION OF THE CDNA ENCODING αIIB AND β3 IN NORMAL HORSES AND TWO HORSES WITH GLANZMANN THROMBASTHENIA

Abstract

Glanzmann thrombasthenia (GT) is an autosomal recessive bleeding disorder described in human patients and dogs that is caused by a quantitative or qualitative deficiency in the platelet glycoprotein complex IIb-IIIa (integrin αIIbβ3). A mutation in either of the genes encoding the αIIb or β3 subunits can result in GT. The purpose of this study was to determine the normal cDNA sequences encoding the αIIb and β3 subunits in horses, compare the normal equine cDNA sequences to cDNA sequences in people and dogs and cDNA sequences obtained from 2 horses (horse 1 and horse 2) previously described with GT, and finally, to identify the molecular defect(s) responsible for GT in these two horses.

Normal equine cDNA sequence encoding αIIb had 82% and 87% identity with human and canine sequences, respectively, while cDNA sequence encoding β3 had 92% and 93% identity with human and canine sequences. The cDNA sequence encoding αIIb from both horses with GT had a single guanine to cytosine substitution at codon 41, nucleotide position 122, in exon 2, which would lead to an amino acid substitution of a proline for an arginine (R41P). This change was not found in individual cDNA

sequences obtained from 10 normal horses. When genomic DNA sequences were evaluated, horse 1 was homozygous while horse 2 was heterozygous for this change, suggesting that horse 2 is a compound heterozygote and likely has another mutation resulting in lack of expression of mRNA. This report represents the first characterization of cDNA sequences encoding for platelet αIIb and β3 in normal horses and in two horses with GT.

Introduction

Glanzmann thrombasthenia (GT) is an inherited, intrinsic platelet defect characterized by a quantitative or qualitative change in the platelet glycoprotein complex IIb-IIIa (integrin αIIbβ3). The subunits are encoded by separate genes and both subunits must be expressed for a stable complex to form on the platelet surface; therefore a defect in either gene can result in GT. This disease has been well documented at the clinical and molecular levels in human beings and dogs.[195,205,215,223] (http://sinaicentral.mssm.edu/intranet/research/glanzmann/menu)

Glanzmann thrombasthenia recently was described in two horses with histories of chronic, intermittent epistaxis that was unrelated to exercise.[224] Horse 1 was a four-year old American Quarter Horse mare located in Auburn, Alabama, while horse 2 was a seven-year old Thoroughbred cross gelding located in Hatfield, Hertfordshire, England. These animals had normal coagulation screening test results, normal platelet numbers, and normal von Willebrand factor antigen levels. Clot retraction and platelet aggregation responses were markedly impaired. Flow cytometric studies indicated a reduction in the αIIbβ3 integrin on platelet surfaces. Based on these findings, both horses were diagnosed

with GT. The purpose of this study was to determine the cDNA sequences encoding normal equine αIIb and β3 and compare them to established human and canine cDNA sequences and to cDNA sequences determined in both affected horses.

Materials and Methods

Platelet-rich plasma was obtained by centrifugation of 100-200 ml of EDTA-anticoagulated whole blood as previously described.[218] Platelets were counted and concentrated into pellets containing ~5 x 10^9 platelets per pellet. Total RNA was harvested from platelet pellets using the Micro to Midi Total RNA Purification System (Invitrogen Corporation, Carlsbad, CA). First strand cDNA synthesis was performed using the Superscript™ First-Strand Synthesis System for RT-PCR using oligodT primers (Invitrogen Corporation).

Genomic DNA was harvested from EDTA-anticoagulated whole blood using the QIAamp® DNA Blood Mini Kit (Qiagen, Incorporated, Valencia, CA). Most primers were designed based on homologous regions of canine and human DNA sequences for αIIb and β3.[178,180] (GenBank accession nos. AF153316 and AF116270). Normal equine cDNA sequence was used to design some of the primers as equine sequence was determined during the course of the study.

Overlapping DNA segments were amplified by polymerase chain reaction using normal equine cDNA or genomic DNA as templates (Tables I and II). The polymerase chain reactions were then repeated using cDNA or genomic DNA isolated from horse 1 and horse 2. Genomic DNA from relatives of horse 1 (dam, sire, and two half-siblings) also was amplified using selected primer sets. Amplification products were separated via

electrophoresis on 1.5% agarose gels. DNA was extracted from target bands using the QIAquick Gel Extraction Kit (Qiagen, Incorporated). Amplicons were sequenced directly by a service laboratory using the same primers used for amplification and an ABI 3100 Genetic Analyzer. Nucleotide and amino acid sequences from different species were compared using the AlignX program of the VectorNTI suite (Informax Invitrogen Life Science Software, Frederick, MD).

Results

Normal equine cDNA sequence encoding αIIb had 82% identity with human and 87% identity with canine sequences. At the amino acid level, percent identities were 82% and 81% respectively. The cDNA encoding β3 in horses had 92% identity with human and 93% identity with canine sequences. Amino acid identity for β3 was 95% when compared to both human and canine sequence. Within the four calcium-binding domains of αIIb equine cDNA exhibited 86-88% identity with human nucleotide sequence (Figure 5, Table 3). Amino acid identity within the four calcium-binding domains between equine and human ranged from 83-91%. When equine nucleotide sequences were compared to canine sequences within the four calcium binding domains, nucleotide identities ranged from 83-91%, and amino acid identities ranged from 75-100%. cDNA sequences for equine αIIb and β3 have been submitted to GenBank (accession nos. AY322154 and AY999064).

cDNA sequences encoding β3 in horses 1 and 2 with GT were identical to sequences obtained in normal horses. cDNA sequences encoding αIIb also were identical except for a single guanine to cytosine (CGG to CCG) substitution at nucleotide position

122 (codon 41) in exon 2 (Figure 6A and 6B). This change would result in the substitution of a proline for an arginine in the encoded protein. Beginning at the N terminal region, integrin α subunits are composed of seven β-propeller domain repeats, each approximately 60 amino acids in length. The location of the amino acid substitution described here is predicted to be in the first β-propeller domain of αIIb (Figure 6C). Calcium-binding motifs are found in β-propeller domains 4-7.[105,106]

Exon 2 was evaluated by PCR amplification and sequencing using as template genomic DNA from 10 normal horses, from GT horses 1 and 2, and from the sire, dam, and 2 half-siblings of horse 1 that shared the same dam. Genomic DNA sequencing results indicated that horse 2 was homozygous for the single guanine to cytosine base change, while horse 1 was heterozygous for the base change (Figure 7). The genomic DNA sequences encoding exon 2 of 10 normal horses and the dam and two siblings of horse 1 were all identical to cDNA sequences obtained in normal horses. The sire of horse 1 was heterozygous for the base change.

Discussion

Dr. Eduard Glanzmann, a Swiss pediatrician, first described GT in his patients in 1918; however, the association of a deficiency of the platelet glycoprotein complex IIb-IIIa with GT was not made until 1974.[171,198,199] In the decade that followed, this glycoprotein complex was recognized as being the receptor that mediated platelet aggregation and was termed the fibrinogen receptor. In the 1990's it was recognized that the fibrinogen receptor of platelets was an integrin and was designated as αIIbβ3 to comply with the nomenclature for that system. It was recognized at this time that the

subunits were encoded by separate genes and that both subunits were required for a stable complex to form on the platelet surface. The first description of GT at the molecular level in human patients was published in 1990, while the molecular basis for GT in dogs was first reported in 1999.[202,225]

Comparisons of cDNA sequences that encode αIIb and β3 in people and dogs have been reported.[226,227] Equine cDNA sequence encoding αIIb was found to be more similar to canine cDNA (87%) than to human cDNA (82%) although when translated to the amino acid level the percent identity with dog and human were very similar (81 and 82%, respectively). Most murine monoclonal antibodies generated against human αIIb do not cross-react with either the canine or equine platelet integrin subunit (MK Boudreaux, personal observation), likely due to the lack of similarity of the subunits at the amino acid level. Horse cDNA sequences encoding β3 were more similar to human and dog sequences at both the nucleotide and amino acid level, with identities ranging from 92 to 95%. In spite of this similarity, monoclonal antibodies to human β3 that cross-react with the canine subunit do not recognize equine β3 (M.K. Boudreaux, personal observation). The reason for this discrepancy is not known but may be related to conformation the β3 subunit achieves when it is complexed with αIIb on the platelet surface.

Glanzmann thrombasthenia has been well characterized at the functional, biochemical, and molecular levels in people and dogs. In dogs, two different mutations, both in the gene encoding αIIb, have been reported.[203,204] According to the human GT database, maintained by the Mount Sinai School of Medicine, over 60 mutations have been documented in the gene encoding αIIb and over 40 mutations have been documented in the gene encoding β3

(http://sinaicentral.mssm.edu/intranet/research/glanzmann/menu). Over half of the reported cases of GT in human patients involving the gene encoding αIIb are classified as compound heterozygotes. The findings in this study indicate the likely cause of GT in horse 2 is a single nucleotide change at position 122 in Exon 2 of the gene encoding αIIb. While cDNA sequence from horse 1 had the identical base change identified in horse 2, genomic DNA sequence was heterozygous for this base change. This suggests that horse 1 is likely a compound heterozygote. The finding that only the sire and not the dam was heterozygous for this base change further confirms that horse 1 is likely a compound heterozygote. The dam is likely heterozygous for the unidentified mutation. The other unidentified mutation apparently results in either total lack of expression or extreme instability of mRNA coding for αIIb, such that message is not well-represented in RT-PCR products of platelet RNA. Thus, this mutation may be located within the promoter region or within an intron or other non-translated portion of the αIIb gene. However, a nonsense mutation within the coding portion of the gene could also result in instability of the mRNA, via nonsense mediated decay.[228]

The base change documented in Exon 2 of horses 1 and 2 would be predicted to result in the change of encoded amino acid 41 from an arginine to a proline. The marked difference in structure of proline compared to arginine, as well as the presence of two adjacent prolines at positions 40 and 41, would be predicted to result in marked instability of the encoded protein. A similar missense mutation in exon 2 of the gene encoding αIIb has been described in human patients.[229] In this report a single nucleotide change resulted in the substitution of a proline for a leucine at amino acid position 55. Similar to the nucleotide change described in this study, the location of mutation in the

human study is predicted to be within the coding region for the first β-propeller domain.[230] Experiments demonstrated that the mutation resulted in severe impairment of expression of the αIIbβ3 complex on the surface of transfected COS-7 cells. The authors hypothesized that the substituted proline caused an aberrant conformation in the encoded protein that prevented association of αIIb with β3, with ultimate lack of expression of the complex on the surface of cells.[229]

This is the first characterization of cDNA sequences encoding for platelet αIIb and β3 in normal horses and in two horses with GT. Future studies will be aimed at identification of the second mutation in the apparently compound heterozygote horse 1. Horses that have platelet-type bleeding, particularly epistaxis, that do not have thrombocytopenia, vasculitis, or von Willebrand Disease, are candidates for the diagnosis of GT. Molecular-based screening assays, based on molecular defects identified in horses with GT, will greatly facilitate the identification of GT in horses with otherwise unexplained platelet-type bleeding.

Acknowledgements

We thank Dr. J. Taintor, Dr. J. Schumacher, Mrs. D. Czerkawski, Mrs. E. Whatley, Mrs. S. Spencer, Mrs. A. Hall, Mrs. D. Shiver, Mrs. A. Mitchell, and Mrs. K. Worley for their help with this project.

Domain 1

	E	F	D	G	D	L	N	T	T	E	Y	V
Human	GAG	TTC	GAC	GGG	GAT	CTC	AAC	ACT	ACA	GAA	TAT	GTC
	E	F	D	G	N	L	N	T	T	E	Y	V
Dog	GAG	TTC	GAC	GGG	AAT	CTC	AAC	ACT	ACA	GAG	TAT	GTC
	E	F	D	E	D	L	S	T	T	E	Y	V
Horse	GAG	TTC	GAT	GAA	GAT	CTC	AGC	ACT	ACA	GAG	TAT	GTC

Domain 2

	D	V	N	G	D	G	R	H	D	L	L	V
Human	GAC	GTC	AAC	GGG	GAT	GGG	AGG	CAT	GAT	CTG	CTG	GTG
	D	V	N	G	D	G	R	H	D	L	L	V
Dog	GAT	GTC	AAC	GGA	GAC	GGG	CGG	CAC	GAC	TTG	CTG	GTG
	D	V	N	G	D	R	R	H	D	L	L	V
Horse	GAC	GTT	AAC	GGG	GAC	AGG	AGG	CAC	GAC	CTG	CTG	GTG

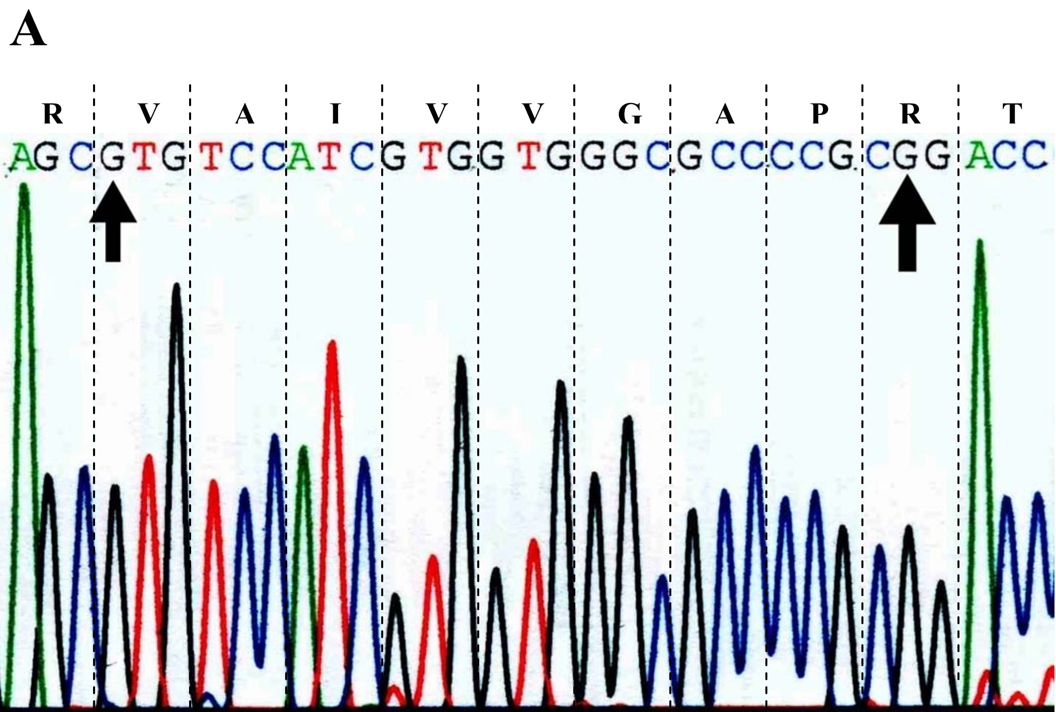
Domain 3

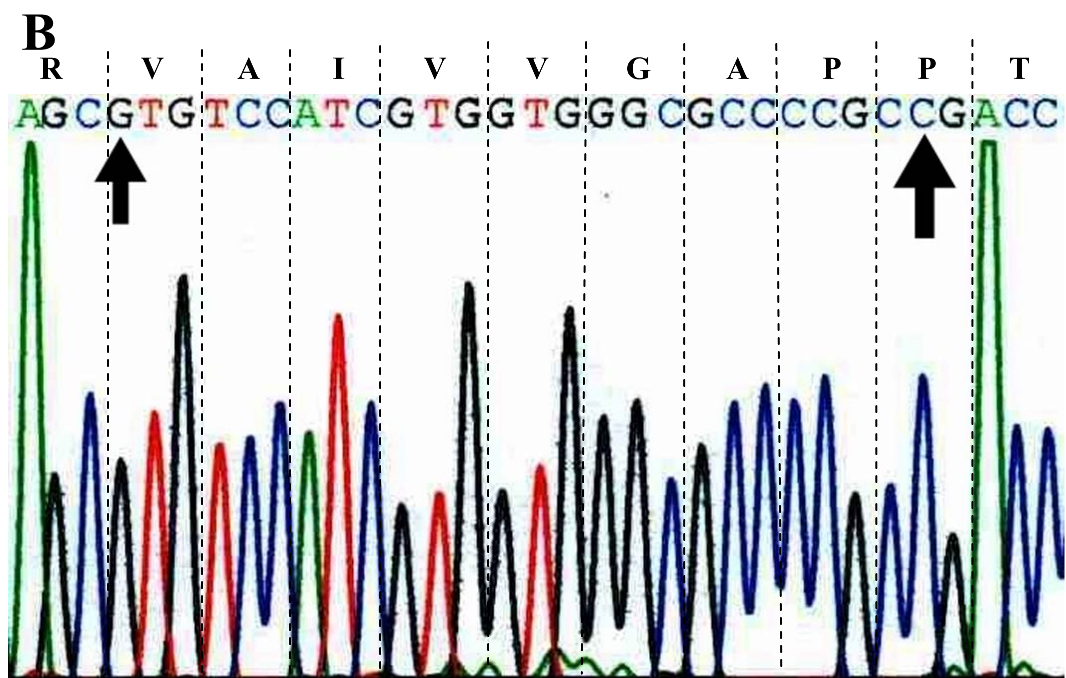
	D	L	D	R	D	G	Y	N	D	I	A	V
Human	GAC	CTC	GAC	CGG	GAT	GGC	TAC	AAT	GAC	ATT	GCA	GTG
	D	L	D	R	D	G	Y	N	D	V	A	V
Dog	GAC	CTC	GAC	CGG	GAC	GGC	TAC	AAC	GAT	GTT	GCA	GTG
	D	L	N	R	D	G	Y	N	D	V	A	V
Horse	GAC	CTC	AAC	CGA	GAT	GGC	TAC	AAT	GAT	GTT	GCA	GTG

Domain 4

	D	I	D	D	N	G	Y	P	D	L	I	V
Human	GAC	ATC	GAT	GAC	AAC	GGA	TAC	CCA	GAC	CTG	ATC	GTG
	D	I	D	D	N	G	Y	P	D	L	L	V
Dog	GAC	ATC	GAT	GAC	AAT	GGA	TAC	CCA	GAC	CTA	CTG	GTA
	D	I	D	D	N	G	Y	P	D	L	L	V
Horse	GAC	ATC	GAT	GAC	AAT	GGA	TAT	CCA	GAC	CTA	TTG	GTG

Figure 5. Comparison of human, dog, and horse nucleotide and predicted amino acid sequences of the 4 calcium binding domains with platelet integrin subunit αIIb. Differences among species are shown in black boxes.


A
R V A I V V G A P R T
AGCGTGTCCATCGTGGTGGGCGCCCCGCGGACC

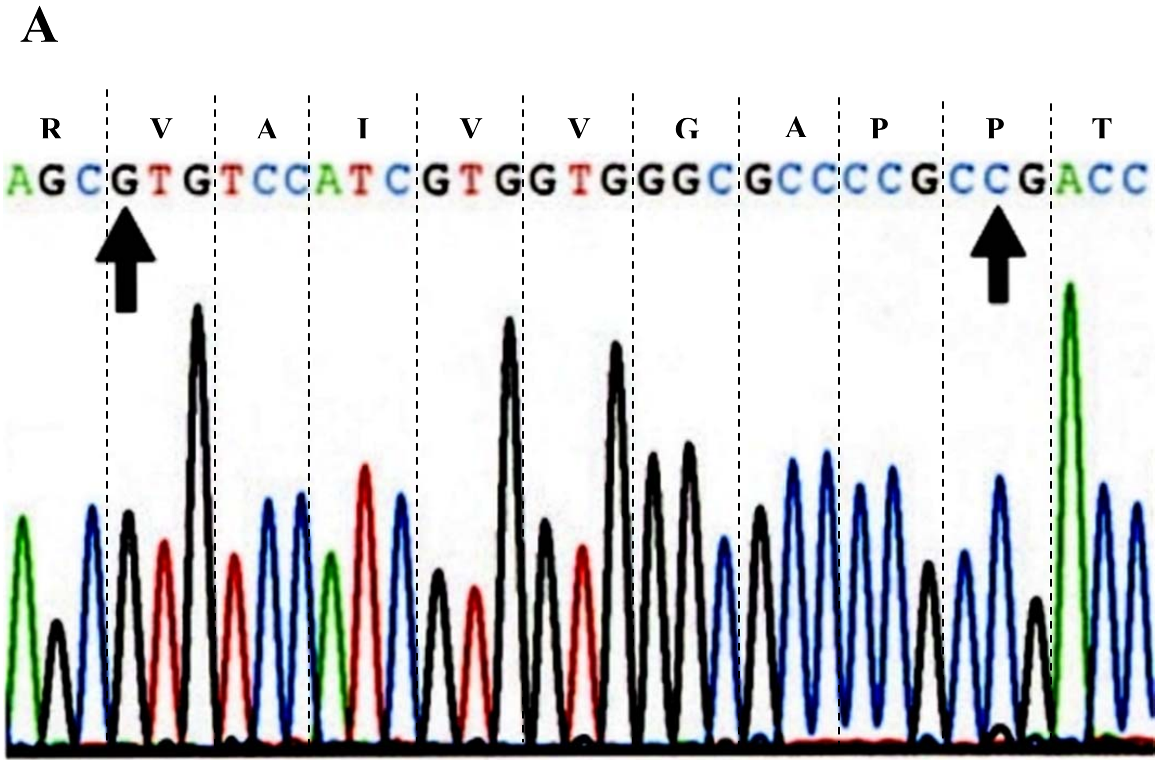

B
R V A I V V G A P P T
AGCGTGTCCATCGTGGTGGGCGCCCCGCCGACC

C

```
Human    LNLDPVQLTFYAGPNGSQFGFSLDFHKDSHGRVAIVVGAP
Canine   LNLDPVQLTFYTGPNGSHFGFSLDFYKDNHGRVAFVVGAP
Equine   LNLDPVQLTFYTGPNGSHFGFSLDFYKDSHGRVAIVVGAP

Human     RTLGPSQEETGGVFLCPWRAEGGQC
Canine    RTLGRSQEETGGVFLCPWRAEGGQC
Equine    RTLGRSQEETGGVFLCPWRAEGGQC
```

Figure 6. cDNA sequences encoding the beginning of exon 2 of αIIb with corresponding amino acids from a normal horse (**A**) and a horse with Glanzmann thrombasthenia (**B**; horse 2) along with amino acids composing the first β-propeller domain of αIIb (**C**). In both nucleotide sequences, the first arrow indicates the first nucleotide of the first complete codon of exon 2, and the second arrow indicates nucleotide position 122 at codon 41. **A.** The second arrow in the sequence indicates a G at nucleotide position 122 in the normal horse. **B.** The second arrow in the sequence indicates a change of G to C at nucleotide position 122, with the encoded amino acid changed from an arginine (R) to a proline (P) Horse 1 cDNA sequence was identical to horse 2 at this location. **C.** The length of the first β-propeller domain of αIIb has been estimated at ~65 amino acids.[230] Normal human, canine, and equine amino acids are provided for comparison; normal sequence variations are noted with solid black boxes. The conserved amino acid changed from an arginine to a proline by the single base change found in horses 1 and 2 is demarcated by a clear box.


A
R V A I V V G A P P T
AGCGTGTCCATCGTGGTGGGCGCCCCGCCGACC

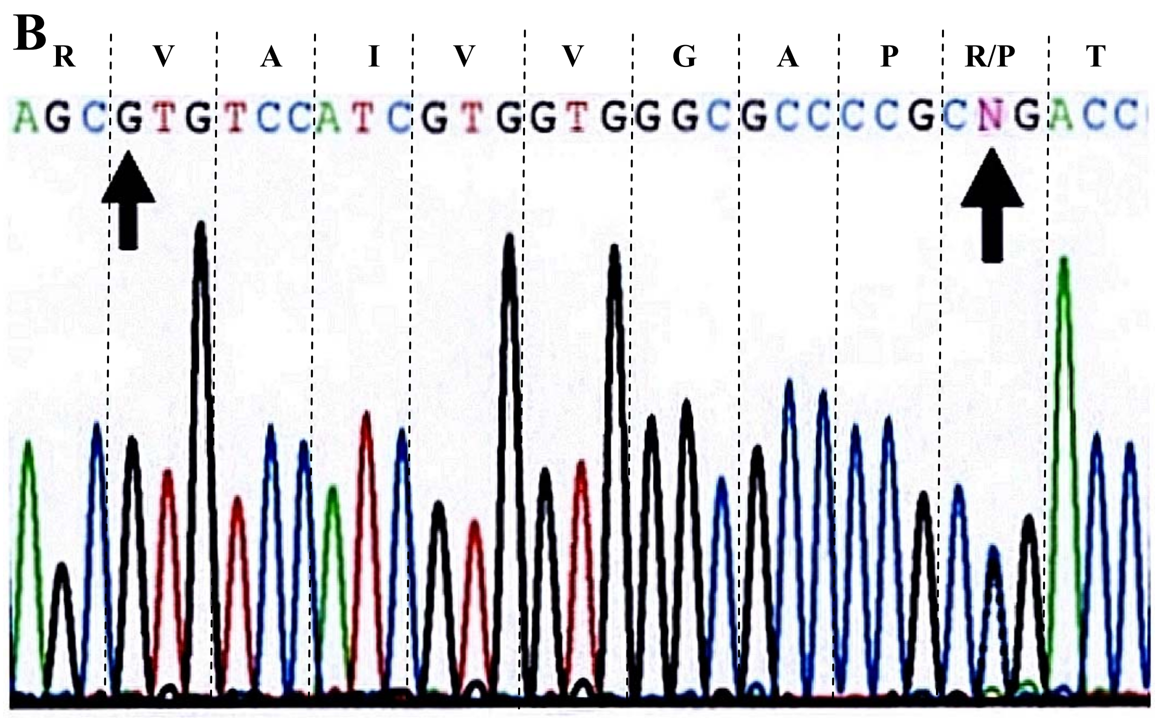

B
R V A I V V G A P R/P T
AGCGTGTCCATCGTGGTGGGCGCCCCGCNGACC

Figure 7. Genomic DNA sequence encoding the beginning of exon 2 of αIIb in two horses with Glanzmann thrombasthenia (horse 2 sequence in **A**, horse 1 sequence in **B**) with amino acid sequence corresponding to part of the first β-propeller domain. In both sequences, the first arrow indicates the first nucleotide of the first complete codon of exon 2, and the second arrow indicates nucleotide position 122 at codon 41. **A**. The second arrow indicates the nucleotide change of G to C in exon 2 of the αIIb gene. The encoded amino acid is changed from an arginine to a proline. This horse was homozygous for the cytosine for guanine substitution. **B.** The second arrow indicates the presence of both C and G nucleotides (designated as N) in exon 2 of the αIIb gene. This horse and the sire were heterozygous at this location. The dam and two half-siblings were homozygous for the normal sequence and phenotypically normal.

Table 1. PCR conditions used to amplify equine αIIb cDNA

Primer Name	Primer Sequence 5' to 3'	PCR Conditions*	Target (bp)
PreEx 1 f Exon 5 r	ATTCCTGCCTGGGAGGTTGTG GCCGGCTTCGCAGTAGCGCTT	30 sec, 30 sec, 45 sec anneal 55°C, 40 cycles	634
Exon 4 f Exon 12 r	ACGCCCGTAGGTGGCTGCTTT GTAGCCATCTCGGTTGAGGTC	30 sec, 30 sec, 45 sec anneal 55°C, 40 cycles	741
Exon 12 f Exon 15 r	CTGACTGGCACACAGCTCTAT CACAGCAGGATTCAGCGAATC	30 sec, 30 sec, 45 sec anneal 50°C, 40 cycles	372
Exon 14 f Exon 21 r	CTACTGGTAGGAGCTTATGGG CCAGCTCACACAGCACTATCT	30 sec, 30 sec, 45 sec anneal 45°C, 40 cycles	764
Exon 21 f Exon 29 r	GAGGCTCATCTGTGACCAGAA CAGCACCCACCAGATGGGAAT	30 sec, 30 sec, 45 sec anneal 53°C, 30 cycles	903
Exon 28 f UTR r	ACAGTCACGCGCTTGGTTCAA AGAATAGTGTAGGCTGCACCA	30 sec, 30 sec, 45 sec anneal 51°C, 30 cycles	285

bp, base pairs; f, forward; r, reverse; sec, seconds; UTR, untranslated region

* With all PCR conditions, denaturation was at 94°C and extension at 72°C for the indicated number of cycles.

Table 2. PCR conditions used to amplify equine β3 cDNA.

Primer Name	Primer Sequence 5' to 3'	PCR Conditions*	Target (bp)
PreEx A f Ex B r	CCGCGGGAGGCGGACGAGAT CTGGCCGGAGCCGGAGTGCAA	30 sec, 30 sec, 45 sec anneal 58°C, 40 cycles	360
Ex A f Exon C r	GGCCCAACATCTGTACCACGC GGCCTCTGGTGGGGAGATAAA	30 sec, 30 sec, 45 sec anneal 54°C, 40 cycles	510
Exon C f Exon F r	GCTTTTGTGGACAAGCCTGTG CTGGGAGAGCTTCTCTGTCAT	30 sec, 30 sec, 45 sec anneal 51°C, 35 cycles	440
Exon D f Exon H r	AAGCAGAGTGTGTCACGGAAC CAGCTCCACTTTAGAGCGGAT	30 sec, 30 sec, 45 sec anneal 52°C, 40 cycles	447
Exon G f Exon I r	CTCATCCCCGGAACCACAGTG CTGAGCACATCTCCCCCTTGTA	30 sec, 30 sec, 45 sec anneal 55°C, 30 cycles	642
Exon I f Exon J r	CCATCAAGCCTGTGGGCTTCA GCGCGTGGTACAGTTGCAGTA	30 sec, 30 sec, 30 sec anneal 55°C, 30 cycles	446
Exon I2 f Exon M r	TACAAGGGGGAGATGTGCTCA AAAGCAGGACCACCAGGATGT	30 sec, 30 sec, 45 sec anneal 51°C, 40 cycles	505
Exon K f Exon N r	GCCCTCTACGAGGAGAATACC AGTGCCCCGGTAGGTGATGTT	30 sec, 30 sec, 45 sec anneal 51°C, 35 cycles	420
Exon N f UTR r	GCCACATCCACCTTCACCAAC CAGCCCTGCCCTTCTCTTCAG	30 sec, 30 sec, 30 sec anneal 56°C, 40 cycles	429

bp, base pairs; f, forward; r, reverse; sec, seconds; UTR, untranslated region.

* With all PCR conditions, denaturation was at 94°C denature and extension at 72°C for the indicated number of cycles.

Table 3: Identity of equine cDNA with human and canine sequences using cDNA encoding the four calcium-binding domains (CBD) and entire structure of αIIb.

Equine cDNA	% nucleotide identity with human	% amino acid identity with human	% nucleotide identity with canine	% amino acid identity with canine
CBD 1	86	83	86	75
CBD 2	88	100	83	100
CBD 3	88	83	88	91
CBD 4	88	91	91	100
Entire αIIb	82	82	87	81

CHAPTER IV.

A 10-BASE-PAIR DELETION IN THE GENE ENCODING PLATELET GLYCOPROTEIN IIB ASSOCIATED WITH GLANZMANN THROMBASTHENIA IN A HORSE

Abstract

Glanzmann thrombasthenia (GT) is an autosomal recessive bleeding disorder that is caused by a quantitative or qualitative deficiency in the platelet membrane glycoprotein complex αIIbβ3, also known as the fibrinogen receptor. Mutations in either of the genes encoding the αIIb or β3 subunit can cause GT. A four-year-old American Quarter Horse mare from Auburn, Alabama with a history of chronic epistaxis was diagnosed with GT. A previous study identified a single nucleotide change, causing an amino acid substitution in exon 2 of the cDNA encoding αIIb. Analysis of genomic DNA showed that the horse was heterozygous for this change. Based on these findings and the mode of inheritance of GT, it was theorized that this horse was a compound heterozygote, and that the mutation in the second allele was causing either a lack of mRNA synthesis or nonsense-mediated decay. Sequencing of the non-protein-coding areas of the αIIb gene in the affected horse showed that one allele had a 10-base-pair deletion that included the splice site between exon 11 and intron 11. This mutation would result in lack of splicing between exons 11 and 12 and introduction of a premature stop codon 50 base pairs

downstream of the mutation. The dam and a half-sibling of the affected horse were found to be heterozygous for this mutation; the sire is heterozygous for the exon 2 mutation and negative for the 10-base pair deletion. These findings document a second, distinct mutation in the gene encoding αIIb as a cause for GT in horses.

Introduction

Glanzmann thrombasthenia (GT) is an inherited platelet function disorder that has been described in people, dogs, and horses.[195,205,214,215,224] GT is the result of a quantitative or qualitative reduction in the platelet fibrinogen receptor (also termed platelet glycoprotein complex IIb-IIIa or integrin αIIbβ3). Clinical signs usually are characterized by cutaneous and mucosal bleeding and may include purpura, epistaxis, gastrointestinal hemorrhage and gingival bleeding. The disease was first described in 1918 by Swiss pediatrician Dr. Eduard Glanzmann.[194] The realization that GT was caused by a deficiency in the glycoprotein IIb-IIIa complex was not reached until 1974.[162,171,198] Later discoveries showed that this receptor was responsible for platelet aggregation via binding of fibrinogen.[69,231] Additional studies indicated that both receptor subunits (αIIb and β3) are required for stable assembly of the receptor on the platelet surface.[97] Separate genes encode the subunits, and a mutation in either gene can result in GT.

The first descriptions of the molecular basis for GT in people and dogs were published in 1990 and 1999 respectively.[202,225] Many different mutations have been documented to cause GT in human patients.[202,225] Two mutations have been documented to cause GT in dogs.[203,204] We recently described the first identified mutation causing GT

in horses, a single guanine to cytosine substitution in codon 41 in exon 2 of the gene encoding the αIIb subunit, resulting in an arginine to proline substitution.[232]

The purpose of the present report is to describe a second mutation causing Glanzmann thrombasthenia in a 4-year-old Quarter Horse mare that had been diagnosed with GT.[224] In our previous study, the affected horse was found to be heterozygous for the mutation in exon 2 described above and we concluded the horse was a compound heterozygote.[232] The second mutation, which resulted in a lack of detectable mRNA from the other allele in this compound heterozygote, was theorized to be causing either a lack of mRNA synthesis or nonsense-mediated decay.[228,232] Therefore, our next step was to evaluate the non-protein-coding areas of the αIIb gene in this horse.

Materials and Methods

Genomic DNA from normal horses and the affected horse was isolated using the QIAamp® DNA Blood Mini Kit.[b] from whole blood collected in EDTA. Primers were designed from cDNA sequence of the horse αIIb gene (GenBank accession number AY322154) and normal equine genomic DNA sequence (unpublished data). Intron length was predicted from known canine (GenBank accession number AF153316) and human DNA sequence for αIIb.[178] Polymerase chain reactions were performed and electrophoresis was used to separate PCR products on 1.5% agarose and 2.5% metaphor® agarose gels.[c] The QIAquick Gel Extraction Kit[b] was used to extract DNA from target bands. Harvested DNA was sequenced using primers used for PCR by the Auburn University Genomics & Sequencing Lab using an ABI 3100 Genetic Analyzer. The

sequence of some selected portions of genomic DNA isolated from the sire, dam, and 2 half-siblings of the affected animal was also determined.

Results

The sequence of fragments amplified from genomic DNA including 1877 nucleotides 5' to the translation start codon showed no differences between normal and affected horses (not shown), suggesting that a promoter defect was not the cause of absence of mRNA from the second defective allele in platelets of the affected horse. Furthermore, sequences of fragments amplified from genomic DNA including splice sites and entire introns 2-10 and 13, and parts of introns 1 and 12 near exons, revealed no differences between normal and affected horses (not shown). However, a difference was identified at the junction between exon 11 and intron 11.

A PCR designed to amplify exon 11 with partial flanking introns (primers: Intron 10 forward- CCATGTCCAGGATCGGGAATT and Intron 11 reverse- CCGAATCTGCTGGTCCATCTT) produced a single, 241-base pair (bp) product using DNA from normal horses as template (Figure 8, Lane 2). A similar amplification using affected horse DNA as template produced 2 bands near the target size, 1 similar in electrophoretic mobility to that from the normal horses and 1 with an electrophoretic mobility corresponding to approximately 230 bp (Figure 8, Lane 3). The 2 bands generated by DNA from the affected horse were too close together to harvest as distinct products for sequencing analysis, so they were sampled using a band stab technique.[233] Briefly, each band was stabbed with a hypodermic needle; the needle was then dipped into a tube containing a fresh PCR reaction mixture, and the DNA was reamplified.

Reamplified products produced from each band using the band stab technique were clearly distinguishable when viewed on a MetaPhor® agarose gel (not shown). This method allowed for harvesting distinct products that could be submitted for sequencing.

The 240-bp product from the affected horse showed a sequence identical to normal horse throughout the fragment (Figure 9A and not shown). Analysis of the sequence from the other product (230 bp) from the affected horse showed a 10-base-pair deletion including the splice site between exon 11 and intron 11 (Figure 9B). This mutation would be predicted to result in lack of splicing of intron 11 and inclusion of a premature stop codon 50 bp downstream of the mutation in the incompletely spliced mRNA. Identical PCR performed on genomic DNA from the sire, dam, and 2 half-siblings indicated that the dam and 1 of the half-siblings were heterozygous for this deletion.

Discussion

Glanzmann thrombasthenia has been well characterized at both the clinical and molecular level in people and dogs. In human patients, there have been over 100 identified mutations associated with GT. The majority of them involve the αIIb gene, and many people with GT are compound heterozygotes.[a] Both mutations associated with GT that have been identified in dogs are in the gene encoding αIIb.[203,204] One of the mutations results in a splicing defect of intron 13.[203]

In this study, we describe a 4-year-old American Quarter Horse mare with GT that is a compound heterozygote. The first mutation, in exon 2 of the gene encoding αIIb, was described in a previous paper.[232] The sire of the GT horse is heterozygous for the

exon 2 mutation and is asymptomatic.[232] The mutation described here, a 10-base-pair deletion including the splice site at the end of exon 11, likely results in little or no expression of mRNA from this copy of the αIIb gene because cDNA sequence from the affected gene could not be detected.[232]

Because no promoter defects were identified, the most likely reason for the absence of mRNA from the gene with the 10-bp deletion in platelets is nonsense-mediated decay (NMD) of mRNA. NMD prevents translation of truncated proteins that can often act in a dominant-negative manner.[234] Normally, pre-mRNA is transcribed and then processed, which includes the removal of introns. During splicing, exon junction complexes are deposited 20-24 nucleotides upstream of exon-exon junctions. These complexes remain with the mRNA until the message undergoes initial translation.[235,236] Exon junction complexes downstream of premature stop codons trigger NMD.[236] The absence of exon junction complexes downstream of stop codons in the terminal exon at the 3' end of the mRNA helps distinguish normal translation stop codons from premature stop codons.[236] Pre-mRNA from the copy of the αIIb gene with the 10-base-pair deletion described in this report likely does not undergo splicing between exons 11 and 12. This results in a premature stop codon in the intron 11 sequence remaining in the message. Exon junction complexes deposited during removal of introns downstream of this premature stop codon would trigger degradation of the mRNA, consistent with its absence in platelets.

This study further characterizes the molecular basis for Glanzmann thrombasthenia in horses. Although these findings show a strong association between the 10-base-pair deletion described here and GT, breeding experiments involving clinically

normal heterozygotes would be needed to prove a fundamental relationship between this mutation and the clinical presentation of GT. Still rarely recognized, horses with GT have been diagnosed on at least 3 continents.[216,217,224,237] Glanzmann thrombasthenia should be a differential diagnosis for horses with platelet-type bleeding, especially epistaxis, that are not thrombocytopenic and do not have von Willebrand disease. Future research will focus on the development of molecular screening assays to identify horses that are either homozygous or heterozygous for mutations resulting in GT.

Footnotes

[a]http://sinaicentral.mssm.edu/intranet/research/glanzmann/menu

[b]Qiagen, Inc., Valencia CA

[c] BioWhittaker Molecular Applications, Rockland, ME

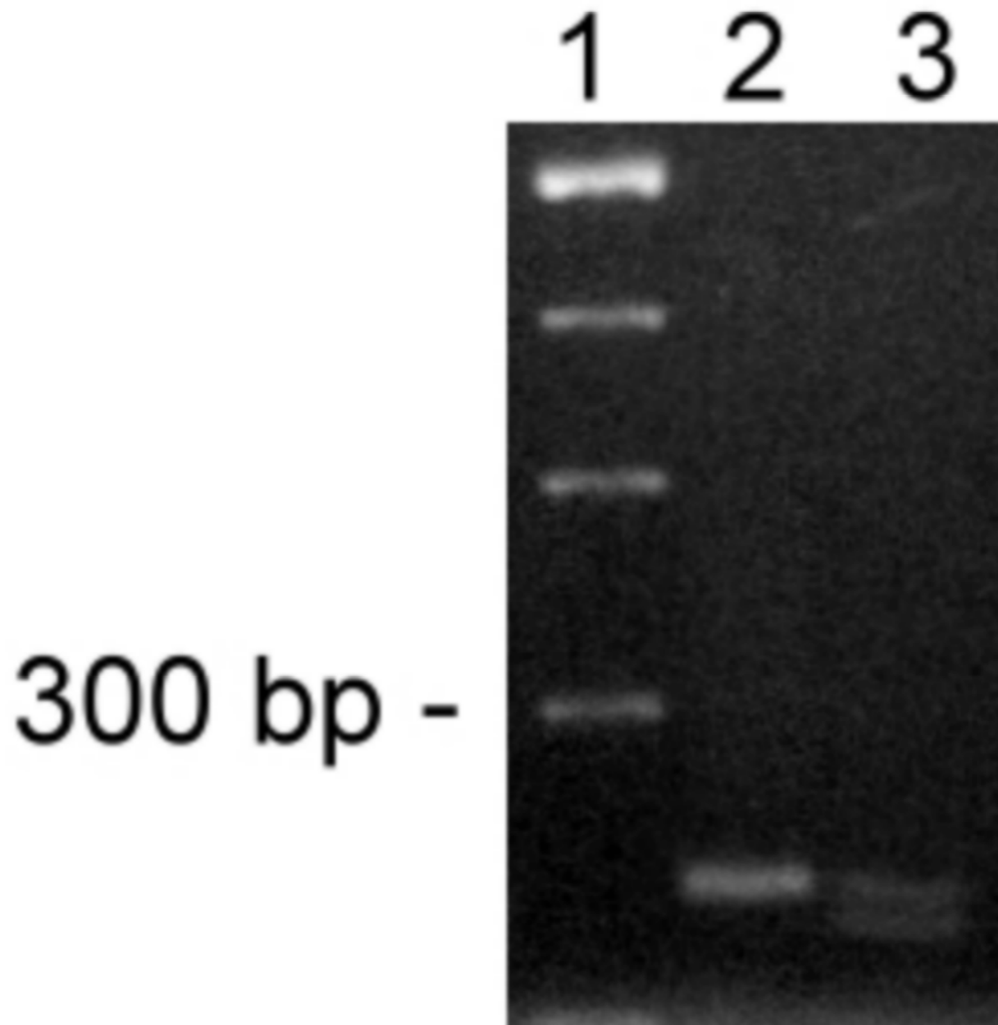


Figure 8. A 2.5% MetaPhor® agarose gel of products from a PCR designed to amplify exon 11 with partial flanking introns in the gene encoding αIIb. Lane 1: molecular ladder. Lane 2: PCR product amplified from normal horse DNA template. Lane 3: product amplified from DNA from the affected horse.

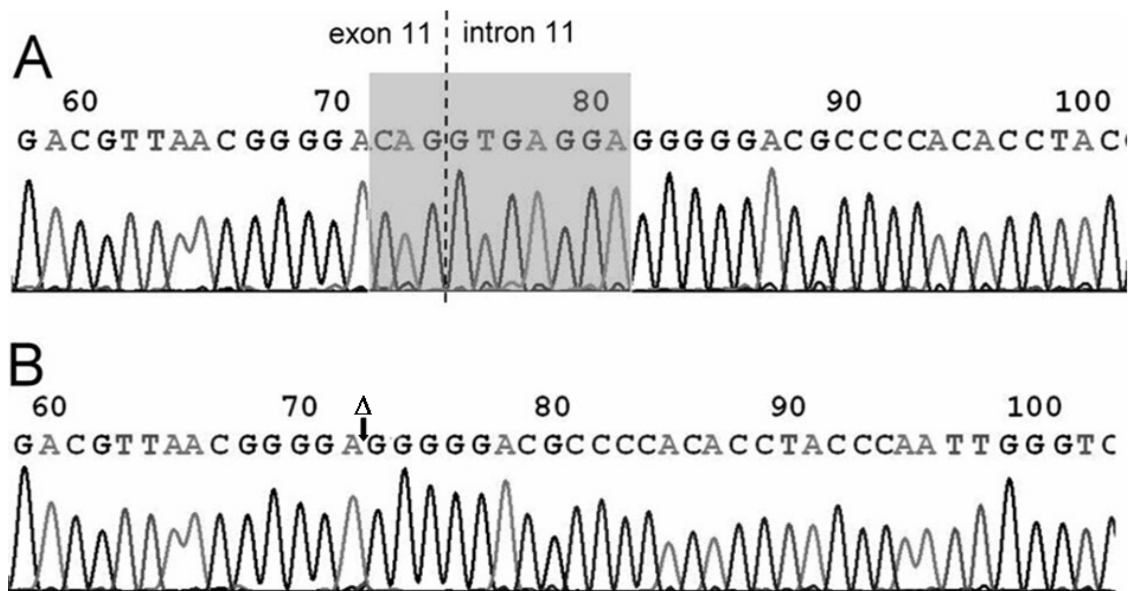


Figure 9**.** Sequence data from the products of PCR reaction amplifying exon 11 and parts of flanking introns in the αIIb gene of a horse with Glanzmann thrombasthenia. **A. 240-bp PCR product.** The dotted line indicates the end of exon 11 and beginning of intron 11 in the gene encoding αIIb. The shaded area shows the 10 base pairs that are deleted in the other copy of the gene (shown in B). The sequence of this fragment is identical to normal horse. However, the gene contains a mutation in exon 2. **B. 230-bp PCR product.** Δ indicates the position of the 10 base pair deletion that includes the splice site at the end of exon 11 and the beginning of intron 11 in the gene encoding αIIb.

CHAPTER V.

REDUCED SURFACE EXPRESSION OF INTEGRIN αIIBβ3 IN CELLS TRANSFECTED WITH EQUINE CDNA CONTAINING A SINGLE BASE CHANGE ASSOCIATED WITH EQUINE GLANZMANN THROMBASTHENIA

Abstract

Glanzmann thrombasthenia (GT) is an inherited intrinsic platelet disorder described in human beings, dogs and horses caused by a quantitative or qualitative reduction in the integrin αIIbβ3 (glycoprotein complex IIb-IIIa) on the surface of platelets. This disease has been well-documented in people and dogs, and two mutations associated with equine GT recently have been identified. One is a single base substitution in exon 2 of the gene encoding αIIb resulting in a proline substituted for an arginine. The purpose of this study was to establish the functional importance of the exon 2 mutation identified in horses and to identify an antibody specific to αIIb that would cross react with equine αIIb.

In our experiments, an antibody recognizing human αIIb was shown to cross-react with equine platelets. Transfection of COS-7 cells with plasmid constructs containing full-length cDNA cloned from wild type equine αIIb and wild type human β3 (wt/wt) and a separate transfection using constructs containing full-length cDNA cloned from mutant equine αIIb and wild type human β3 (mu/wt) were performed. At 48 hours, wt/wt transfected cells had increased fluorescence compared to mu/wt transfectants when

labeled with antibodies to αIIb and the αIIbβ3 complex. The fluorescence of mu/wt transfected cells was still significant when compared to negative control cells. These findings provide evidence that the exon 2 mutation may result in partial functionality of the αIIb protein in horses and this mutation results in a form of GT similar to Type II GT in people. Similar transfection experiments have been performed in human medicine to establish that specific mutations result in GT; to our knowledge, this is the first such experiment documented in veterinary medicine.

Introduction

Glanzmann thrombasthenia (GT) is an inherited intrinsic platelet defect that is caused by a quantitative or qualitative reduction in the platelet fibrinogen receptor (also known as glycoprotein complex IIb-IIIa or integrin αIIbβ3). This receptor is made up of two subunits, glycoprotein IIb (αIIb) and IIIa (β3). GT has been described in people, dogs, and recently, horses.[195,205,214,215,224] Clinical signs of this disorder include epistaxis, mucosal bleeding, gastrointestinal hemorrhage, and purpura. Six horses with clinical signs consistent with equine GT have been described, but only three of these cases have been examined at the molecular level with two mutations described.[232,238] One mutation is a single base substitution in exon 2 of the gene encoding glycoprotein IIb. This mutation results in a substitution of a proline for an arginine and would be predicted to result in marked instability of the encoded protein.[232] The second mutation is a 10-base-pair deletion at the junction of exon 11 and intron 11 that likely results in nonsense-mediated decay of the RNA from this copy of the gene.[238] Currently, one Thoroughbred horse has been found to be homozygous for the exon 2 mutation,[232] one Peruvian Paso is

homozygous for the 10-base-pair deletion (unpublished data), and an American Quarter Horse is a compound heterozygote with both mutations present on separate alleles.[232,238]

Over 100 mutations causing GT have been described in human beings (http://sinaicentral.mssm.edu/intranet/research/glanzmann/menu). Many people with GT are compound heterozygotes, and a mutation similar to the equine αIIb exon 2 mutation has been described.[229] Our transfection study was designed to verify the substitution mutation in exon 2 of the gene encoding αIIb as a cause of GT in horses. Further efforts were made to identify an antibody that would cross-react with equine αIIb in order to provide another diagnostic tool for further description of equine GT.

Materials and Methods

Platelet-rich plasma (PRP) was isolated from citrated blood collected in a 9:1 ratio in 3.8% trisodium citrate as previously described.[218] In flow cytometric experiments, 10 µl of PRP were added to 100 µl of buffer.[205] 20 µl of mouse anti-ruminant CD41/CD61, MCA1095, (Serotec, Raleigh, NC), a monoclonal antibody specific to the fibrinogen receptor αIIbβ3 was added to samples of diluted PRP from a GT horse heterozygous for a previously described mutation in the gene encoding αIIb[232] and from a normal horse. (The GT horse is a compound heterozygote with a documented mutation in the other αIIb allele which results in no detectable levels of mRNA).[238] Samples were incubated for 20 minutes. Secondary antibody labeled with fluorescein isothiocyanate (FITC) (Goat $F(ab)_2$ fragment anti-mouse immunoglobulin G), was incubated with the samples for 20 minutes in the dark on ice. Samples were prepared identically for labeling with mouse anti-human CD41 clone 5B12 (Dako, Carpinteria,

CA) FITC-conjugated antibody specific for glycoprotein IIb, except only the second 20 minute incubation was utilized. For the CD41/61 antibody, secondary antibody was added alone to PRP from both subject and control horse to assess non-specific binding. Unlabeled PRP samples were prepared to evaluate autofluorescence along with samples incubated with the glycoprotein IIb antibody.

Full-length cDNA products were amplified via PCR utilizing human and equine-based primers. Templates were cDNA from a normal horse and a horse documented to be homozygous for the exon 2 substitution mutation in the gene encoding αIIb. Wild type and mutant αIIb cDNA were ligated into pcDNA™ 3.3 eukaryotic expression vectors downstream of the CMV promoter and transformed into STABLE-2 bacteria using the pcDNA™ 3.3-TOPO® TA Cloning Kit (Invitrogen™ Carlsbad, California). The wild type human β3 eukaryotic expression plasmid was a gift generously donated by Dr. David Wilcox, Medical College of Wisconsin. Cloned αIIb cDNA was fully sequenced after transformation. Plasmid DNA was isolated and purified using a Qiagen® Endotoxin Free Maxi kit.

COS-7 cells were cultured in L-15 medium with an antibiotic-antimycotic solution (containing penicillin, streptomycin, and amphoterincin B) (Gibco® BRL) and 10% fetal bovine serum (Hyclone). Cells were grown at conditions of 100% humidity at 37°C with 5% CO_2, and the medium was changed every 2 days. COS-7 cells were grown in a 25 cm^2 flask to 90-95 % confluence at the time of transfection. One day before transfection, the medium was changed to medium without antibiotics. Lipofectamine™ 2000 LTX reagent (Invitrogen™) and Opti-MEM reduced serum media (Invitrogen™) were used in transfections. For each transfection reaction, 4 µg of the αIIb and β3

expression plasmids (8 µg total) were diluted in 0.5 ml Opti-MEM without serum and gently mixed. Next, 20 µl of Lipofectamine 2000 was diluted in 0.5 ml Opti-MEM for 5 min at room temperature. Next, diluted DNA was combined with diluted Lipofectamine reagent and incubated for 20 min at room temperature. The mixture was added to 5 ml of medium in the flask of COS-7 cells. The flask was gently rocked and then incubated for 48 hrs at 37°C. Different flasks of cells were transfected with equine wild type αIIb/human wild type β3 and equine mutant αIIb/human wild type β3 expression plasmids.

Transfection efficiency was checked by including 1µg green fluorescent protein (GFP) eukaryotic expression plasmid, along with αIIb and β3 expression plasmid in another flask of COS-7 cells using the same conditions. The GFP transfected cells were wet-mounted on a glass slide and examined under a fluorescent microscope to estimate the efficiency of the transfection by calculating the percentage of GFP positive cells. This was performed by dividing the number of fluorescent cells by the total number of cells counted (100). Cells were prepared for flow cytometry in a manner similar to the handling of PRP samples.

Results

Equine platelet preparations incubated with anti human CD41 (αIIb) antibody showed cross-reaction with the antibody. Platelets from the GT horse exhibited less fluorescence than normal horse platelets but more than unlabeled normal horse platelets (Figure 10).

Sequencing of the selected mutant equine αIIb cDNA clone confirmed the presence of a guanine to cytosine substitution at codon 41, nucleotide position 122, in exon 2, which would lead to an amino acid substitution of proline for an arginine (R41P). Sequenced clones for both wild type and mutant equine αIIb cDNA contained several other base changes besides the desired mutation. Several of these base changes altered the encoded amino acid (results not shown).

At 48 hours after transfection, COS-7 cells co-transfected with wild type αIIb cDNA and β3 wild type cDNA (wt/wt) exhibited ~30-35% transfection efficiency based on green fluorescent protein expression (results not shown) and flow cytometric detection of CD41/61 antibody binding on cell surfaces (Figure 11). In a separate experiment, cells co-transfected with mutant αIIb cDNA and wild type β3 cDNA (mu/wt) had reduced detection by CD41 antibody labeling when compared to wt/wt transfected cells. Both transfectants had higher levels of CD41 antibody fluorescence compared to unlabeled cells (Figure 12).

Discussion

Glanzmann thrombasthenia has been described at the functional, biochemical, and molecular level in people, dogs and recently horses. In dogs, two different mutations, both in the gene encoding αIIb have been reported.[203,204] In human beings, over 60 mutations have been documented in the gene encoding IIb and over 40 mutations have been documented in the gene encoding β3 (http://sinaicentral.mssm.edu/intranet/research/glanzmann/menu). Over half of the human Glanzmann thrombasthenia cases with mutations in the gene encoding αIIb are

compound heterozygotes. The transfection studies performed here support a single nucleotide change at position 122 in exon 2 of the gene encoding αIIb as a cause of GT in horses. This base change would be predicted to result in the change of encoded amino acid 41 from an arginine to a proline. The location of the change is predicted to be in the nucleotide sequence encoding the first of seven β-propeller domains of the αIIb protein. Calcium-binding domains are found in β-propeller domains 4-7.[105,106] The marked difference in structure and non-polar nature of proline compared to arginine, along with the presence of two adjacent prolines at positions 40 and 41, would be predicted to result in marked instability of the encoded protein.[232]

A similar missense mutation in exon 2 of the gene encoding αIIb has been described in people.[229] In this report,[229] a single nucleotide change resulted in the substitution of a proline for a leucine at amino acid position 55, which, like the equine Exon 2 mutation, is within the coding region of the first β-propeller domain.[230] In the human study, experiments similar to the ones performed in our study demonstrated that position 55 mutation resulted in severe impairment of expression of αIIbβ3 on the surface of transfected COS-7 cells. The authors hypothesized that the substituted proline caused an aberrant conformation in the encoded protein that prevented association of αIIb with β3, with ultimate lack of expression of the complex on the surface of platelets.[229]

An important finding in this study is the identification of a CD41 antibody that cross-reacts with equine αIIb. Another CD41 antibody that cross-reacts with equine platelets has also been reported.[237] Previous flow cytometry studies performed in our laboratory on platelets from GT horses utilized only the CD41/CD61 antibody, which exhibited a small amount of binding when compared to positive controls. This finding

was attributed to antibody cross-reaction with the vitronectin receptor on platelet surfaces.[224] Vitronectin receptors are a complex of the αv subunit and the same β3 subunit (glycoprotein IIIa) found in the fibrinogen receptor.[207] In the present study, the CD41 antibody was documented to bind not only to αIIb on equine platelets, but also αIIb expressed on transfected COS-7 cells. Platelet and COS-7 flow cytometric data suggest that the exon 2 mutation results in significantly reduced, but not complete lack of αIIb expression on platelet and cell surfaces secondary to this mutation. Type I, Type II and Variant forms of GT have been identified in people.[195] Our findings are most consistent with Type II GT, but further studies utilizing an isotype control antibody would be needed for confirmation.

Type I GT is typified by patients having less than 5% of the normal levels of αIIbβ3, a negative clot retraction test, low levels of fibrinogen in their alpha-granules and activated platelets that are unable to bind fibrinogen.[195,206] Increased numbers of vitronectin receptors are present on platelet membranes in patients with Type I disease when the mutation involves the αIIb subunit. This is believed to occur because of an increased availability of β3 subunits that normally would have been incorporated into αIIbβ3 complexes.[207]

In Type II GT, which accounts for approximately 14% of the reported human cases, individuals have 10-20% of normal levels of glycoprotein IIb-IIIa, abnormal clot retraction, and low levels of fibrinogen binding and platelet aggregation.[195,206] The mutations in Type II GT usually are found in the β3 gene.[208] The Variant form of GT is typified by αIIbβ3 levels quantified at 50-100% of normal, a normal or absent clot retraction and little or no fibrinogen binding. Similar to Type II GT, Variant GT most

often results from a mutation in the β3 gene, but it is a qualitative platelet defect versus the quantitative defects found in Types I and II.[195,206]

There are several aspects of our study that warrant further investigation. The wild type and mutant equine αIIb clones utilized in transfection experiments contained multiple base changes compared to published equine αIIb cDNA sequence (GenBank accession number AY322154) and the consensus sequence of the cDNA PCR product. While some changes were consistent with insignificant polymorphisms, some could not be completely ruled out as functional mutations. Therefore, we cannot be certain that the lowered expression of αIIb on mu/wt transfected cells was caused solely by the proline for arginine codon substitution in exon 2. A possible reason for the other base changes is incorporation of a non-consensus PCR sequence into plasmids during the ligation reaction. Another possibility is the alteration of the sequence during bacterial replication. Alternatively, if the bacteria used in transformation were able to produce some of the encoded protein, the product could have possibly been toxic to the bacteria, favoring the growth of bacteria containing altered sequence and that were not capable of synthesizing the protein. However, given the detection of αIIbβ3 on the surface of wt/wt transfected cells, this explanation is unlikely. Further studies are needed to clarify these issues. Higher fidelity techniques may be necessary to select more appropriate bacterial clones. Another possible approach would be the use of site-directed mutagenesis techniques to insure the only difference between wild type and mutant αIIb clones is the desired single base change in exon 2.

Additional experiments are necessary to confirm our initial findings and to definitively rule out non-specific antibody binding and aberrant protein expression by

transfectants. These would include repeating flow cytometric studies with the CD41 antibody with a negative isotype control antibody to verify the significance of the FITC-labeling observed in our study. Separate transfections of plasmid construct, wt equine αIIb, mutant equine αIIb and wt human β3 would be performed along with repeating the transfections performed in this study (wt/wt and mu/wt). Cells from each transfection would be labeled with CD41 and CD41/61 antibodies and their negative controls. Non-transfected COS-7 cells would also be analyzed by flow cytometry for auto-fluorescence and non-specific binding by both antibodies.

This study further emphasizes the significance of a previously described mutation associated with Glanzmann thrombasthenia in horses. Future work will be focused on resolving issues described above and screening the general horse population for both identified mutations associated with GT in horses in order to identify the true prevalence of these mutations in the general horse population.

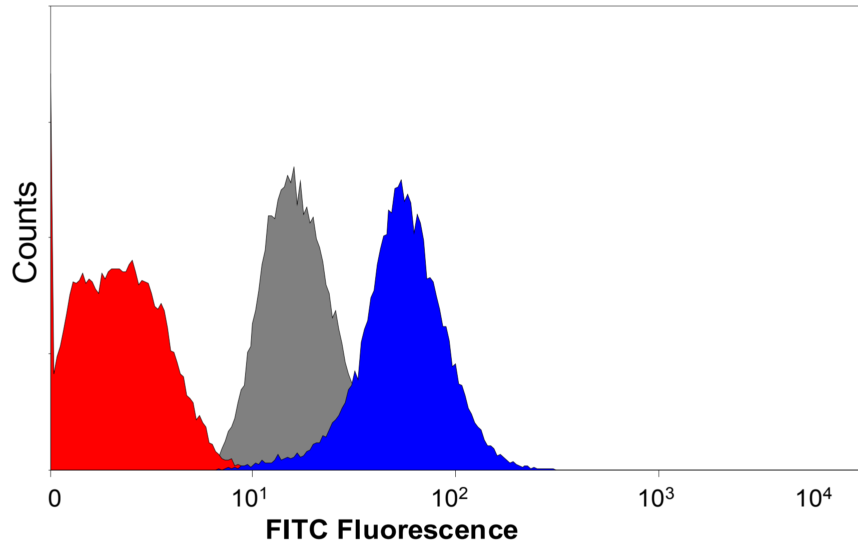


Figure 10. Flow cytometric analysis of CD41 (αIIb) antibody binding to platelets isolated from a normal horse (blue peak) and a compound heterozygote Glanzmann thrombasthenia horse (gray peak) compared to unlabeled normal horse platelets (red peak).

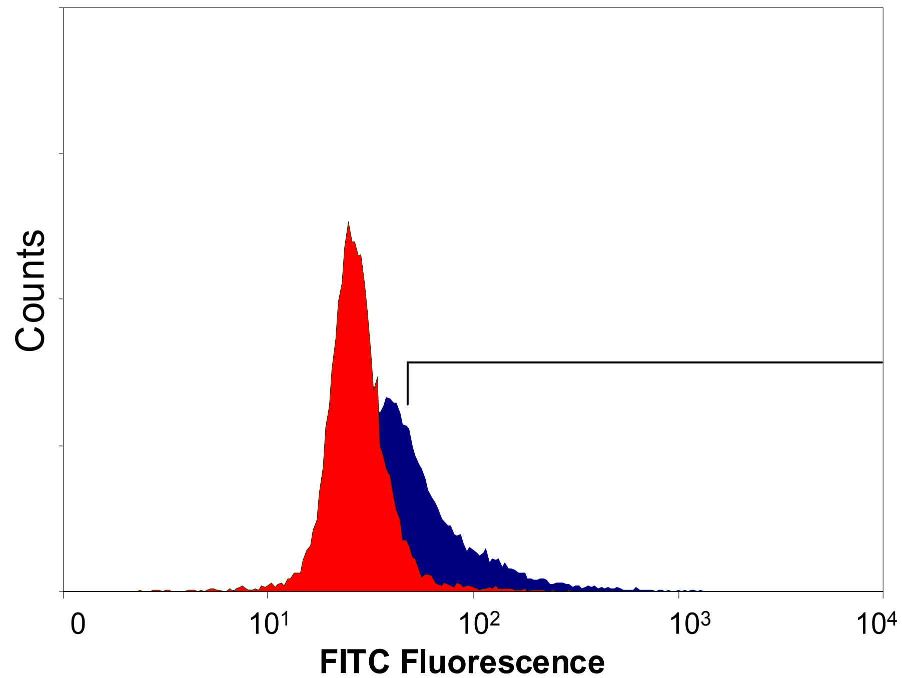


Figure 11. Flow cytometric analysis of CD41/CD61 (αIIbβ3) antibody binding to COS-7 cells transfected with wild type equine αIIb cDNA and wild type human β3 cDNA (wt/wt) and comparison with negative control cells. The red peak represents transfected cells labeled with secondary antibody only and are indicative of non-specific antibody binding. The blue peak represents binding of antibody to transfected COS-7 cells incubated with primary and secondary antibody. Approximately 30-35% of transfected cells were positive (gate for positive is indicated by horizontal line) for CD41/61 antibody binding.

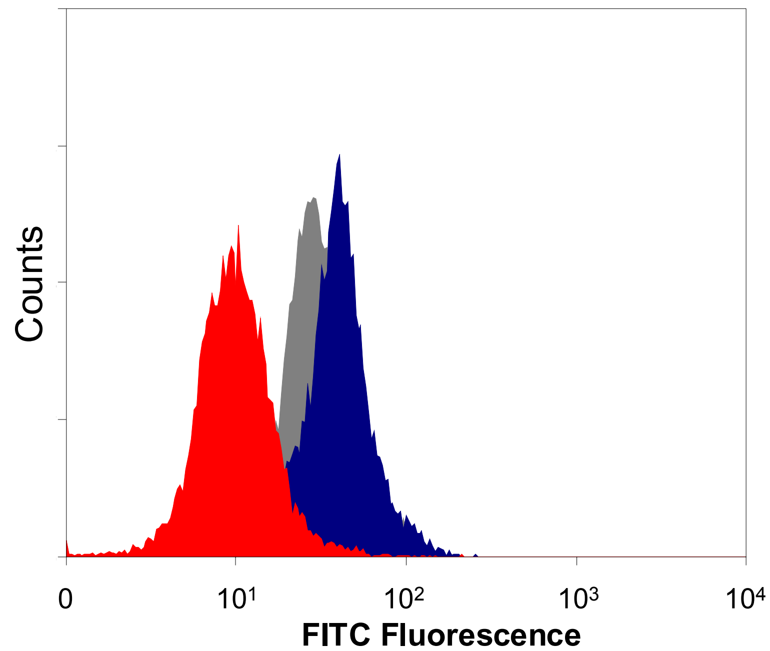


Figure 12. Flow cytometric analysis of CD41 (αIIb) antibody binding to COS-7 cells transfected with wild type equine αIIb cDNA and wild type human β3 cDNA (wt/wt) and cells transfected with mutant equine αIIb cDNA and wild type human β3 cDNA (mu/wt) with comparison to unlabeled transfected (wt/wt) cells. The blue peak represents labeled cells transfected with wild type equine αIIb cDNA and wild type human β3 cDNA (wt/wt). The gray peak represents labeled cells transfected with mutant equine αIIb cDNA and wild type human β3 cDNA (mu/wt). The red peak represents unlabeled wt/wt transfected cells.

CHAPTER VI.

CONCLUSIONS

The purposes of this work were to diagnosis Glanzmann thrombasthenia in two horses and to determine the genetic mutation(s) associated with the disease. One affected horse was heterozygous (horse 1) and the other homozygous (horse 2) for a single nucleotide change at position 122 of exon 2 in the αIIb gene, which resulted in a substitution of a proline for an arginine in the encoded protein. The marked difference in structure of proline compared to arginine, as well as the presence of two adjacent prolines at positions 40 and 41, would be predicted to result in marked instability of the encoded protein. A similar missense mutation in Exon 2 of the gene encoding αIIb has been described in people.[229] That study demonstrated that the mutation resulted in severe impairment of expression of the αIIbβ3 complex on the surface of transfected COS-7 cells. The authors hypothesized that the substituted proline caused an aberrant conformation in the encoded protein that prevented association of αIIb with β3, with ultimate lack of expression of the complex on the surface of platelets.

Using a similar approach, the equine mutation identified in exon 2 was evaluated with transfection of COS-7 cells utilizing equine wild type and mutant αIIb cDNA and human wild type β3 cDNA. Reduced cell surface expression of mutant αIIb and αIIbβ3 complexes detected via flow cytometry supported the hypothesis that this equine

mutation caused reduced expression of the fibrinogen receptor on platelet surfaces. Specific detection of αIIb on cells transfected with mutant αIIb cDNA suggested the cells still expressed low amounts of the protein on their surfaces. This finding was supported by labeling with antibody to the αIIbβ3 complex. These findings suggested this mutation resulted in reduced but not complete loss of expression of the αIIbβ3 integrin on platelets in horses homozygous for this mutation (horse 2 in our study), which was most consistent with Type II GT described in human beings. Horses heterozygous for this mutation and with a normal remaining allele would likely be clinically normal but may or may not have mild detectable reductions in αIIbβ3 on their platelets. Further studies are needed to address this question.

Based on the mode of inheritance of GT, it was theorized that horse 1 was a compound heterozygote, and that the mutation in the second allele was causing either a lack of mRNA synthesis or nonsense-mediated decay. Sequencing of the non-protein-coding areas of the αIIb gene in the second horse showed that one allele had a 10-base-pair deletion that included the splice site between exon 11 and intron 11. This mutation would result in lack of splicing between exons 11 and 12 and inclusion of a premature stop codon, likely triggering nonsense-mediated decay.

There are several important implications of this work. The documentation of two mutations causing GT in horses allows for molecular diagnostic tests for detection of affected and carrier animals. Recently, a Peruvian Paso homozygous for the 10-base-pair deletion mutation was identified (unpublished data). Considering mutations have been found in 3 distinct breeds, which include early breeds (Peruvian Paso, Thoroughbred) that

helped form the foundations for later breeds, at least one of these mutations may be widespread within the equine population.

Although a rarely recognized disease, GT should be a differential diagnosis in cases where horses have platelet-type bleeding (especially epistaxis) and other causes for bleeding such as thrombocytopenia, coagulopathies and von Willebrand disease have been ruled out. Testing of these high risk horses should be done in an attempt to document the prevalence of these mutations in horses. PCR testing could also be used to determine the prevalence of mutations in the general horse population and for carrier detection, which would have consequences for breeding. Additionally, mutations in the $\alpha IIb\beta 3$ integrin gene may have a role in other equine bleeding diatheses such as exercise-induced pulmonary hemorrhage and post-partum bleeding.

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