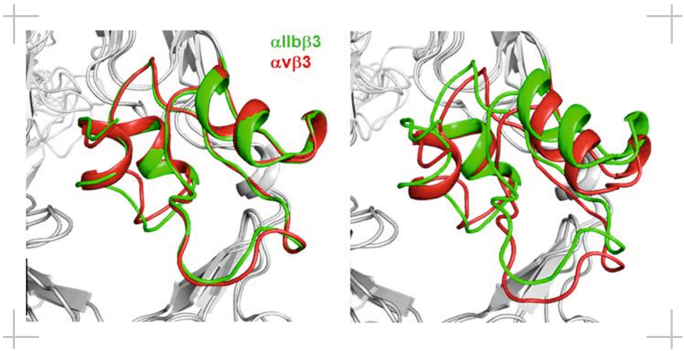
The Role of Antibodies against Endothelial Cells in Immune Mediated Thrombocytopenia

Hevi Wihadmadyatami



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Prof. Dr. med. Ulrich. J. Sachs

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Eingereicht von

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II. Sentot Santoso, **Hevi Wihadmadyatami**, Tamam Bakchoul, Silke Werth, Nadia Al-Fakhri, Gregor Bein, Peter J. Newman, Jieqing Zhu, Volker Kiefel, Behnaz Bayat and Ulrich J. Sachs. Anti-endothelial αvβ3 Antibodies are a Major Cause of Intracranial Bleeding in Fetal/ Neonatal Alloimmune Thrombocytopenia.
Arteriosclerosis, Thrombosis, and Vascular Biology, 2016, volume 36, pages 1517-24.

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Manuscript in preparation

V. **Hevi Wihadmadyatami**, Kathrin Heidinger, Lida Adriana Röder, Silke Werth, Martin Knorr, Gregor Bein, Ulrich J. Sachs and Sentot Santoso. Alloantibody against New Platelet Alloantigen (Lap^a) on GPIIb is Responsible for a Case of Fetal/ Neonatal Alloimmune Thrombocytopenia.
Transfusion, 2015; volume 55, pages 2920-29.

List of Abbreviations

ADP	Adenosin Diphosphate
CHO	Chinese Hamster Ovary cells
cRGD	Cyclic Arginine Glycine Asparagine
ECs	Endothelial Cells
FNAIT	Fetal/ Neonatal Alloimmune Trombocytopenia
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GP	Glycoprotein
GT	Glanzmann's Thrombasthenia
HEK 293	Human Embryonic Kidney 293 cells
HUVEC	Human Umbilical Vein Endothelial Cells
HPA	Human Platelet Alloantigen
HLA	Human Leucocyte Alloantigen
ICH	Intracranial Hemorrhage
IgG	Immunoglobulin G
IVIG	Intravenous Immunoglobulin G
IUPT	Intrauterin Platelet Transfusion
mab	Monoclonal antibody
MAIPA	Monoclonal Antibody Immobilization of Platelet Specific Antigens
PTR	Platelet Transfusion Refractoriness
PTP	Post Transfusion Purpura
Plts	Platelets
ROS	Reactive Oxygen Species
SNPs	Single Nucleotide Polymorphism
vWF	von Willebrand Factor
VEGF	Vascular Endothelial Growth Factor

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1. General Introduction

Platelets are small, enucleated blood cells that play an important role to stop bleeding at the site of disrupted endothelium. This process consists of different steps including platelet adhesion, platelet activation and platelet aggregation (termed primary hemostasis), followed by activation of the coagulation cascade (termed secondary hemostasis). This reaction cascade is maintained and regulated by several receptors expressed on the platelet surface. Recent studies, however, indicated that platelets also contribute to innate and adaptive immunity (Semple et al. 2011). Consequently, low platelet count due to either decreased production or increased destruction (termed thrombocytopenia) and functional platelet dysfunction (termed thrombocytopathy) may not only lead to bleeding disorders but also to other immune mediated diseases (van Ommen & Peters 2012).

The αIIbβ3 integrin represents the most abundant receptor on platelets responsible for platelet adhesion and aggregation mediated by interaction with von Willebrand factor (vWF) and fibrinogen, respectively. In addition, platelets also express αvβ3, which is functionally important as vitronectin receptor for the platelet attachment to endothelium (Gawaz et al, 1997). The αIIb subunit is exclusively expressed in platelets and their precursor megakaryocytes (Grimaldi et al. 1998), whereas the αv and β3 subunits are also found on endothelial progenitor cells (Caiado & Dias 2012), angiogenic endothelial cells (Weis & Cheresh 2011), microglia (Milner 2009), astrocytes (Avalos et al, 2009), cytotrophoblast (Zhou et al. 1997) and syncytiotrophoblast cells of the placenta (Kumpel et al. 2008).

Several studies in the last recent decades documented that platelet reactive antibodies against αIIbβ3 integrin can cause platelet destruction leading to immune mediated thrombocytopenia and bleeding. In addition, the hereditary defect of αIIbβ3 expression on platelets can impair platelet functions associated with the bleeding tendency. However, little is known about the role of αvβ3 and anti-αvβ3 antibodies in this context.

1.1. β3 Integrins

Integrins are a large family of heterodimeric transmembrane glycoproteins, which function as adhesive receptors promoting cell-cell and cell-extracellular matrix interactions. Integrins plays a significant role in several biological processes such as platelet aggregation, inflammation, immune function, wound healing, metastasis, and tissue migration during embryogenesis. Currently, 18 α- and 8 β-subunits form 24 different integrins (Figure 1). Interaction of integrins with their binding partners can induce bi-directional signals, known as *outside-in* and *inside-out* signaling (Hynes 2002; Harburger & Calderwood 2009). This novel signal transduction pathway is not only important for the regulation of ligand binding but also for the modulation of transmembrane protein kinases (e.g receptor tyrosine kinases). Depending on the integrin regulation, specific receptor tyrosine kinases can be recruited which leads to cell growth, cell division, cell survival, cellular differentiation, and apoptosis (programmed cell death).

The β3-integrin family consists of two members, the αIIbβ3 and αvβ3 heterodimers. The αIIbβ3 integrin is only found in platelets and megakaryocytes. On platelets, αIIbβ3 (50.000 - 80.000 copies/platelet) acts as a primary receptor for vWF, fibrinogen and other ligands (vitronectin, fibronectin, and thrombospondin) (Bennett 2005). The α and β subunits are produced independently. In the endoplasmic reticulum, high mannose N-linked oligosaccharides are assembled into the pro-αIIb precursor prior to the formation of pro-αIIbβ3 heterodimer. Subsequently, this complex is transported into the Golgi where pro-αIIb undergoes further processing; conversion of mannose into complex oligosaccharides, and proteolytic cleavage of the pro-αIIb into a disulfide-linked light and heavy chains. Afterwards, the mature form of αIIbβ3 complex is expressed on the cell surface or stored within alpha granules (Floyd & Ferro 2012). The αvβ3 integrin is synthesized by a similar mechanism (Grimaldi et al. 1998). However, other than αIIb, αv subunit does not undergo proteolytic cleavage. Furthermore, αIIbβ3 complex formation is calcium-dependent, whereas αvβ3 is magnesium-dependent (Bray et al. 1988).

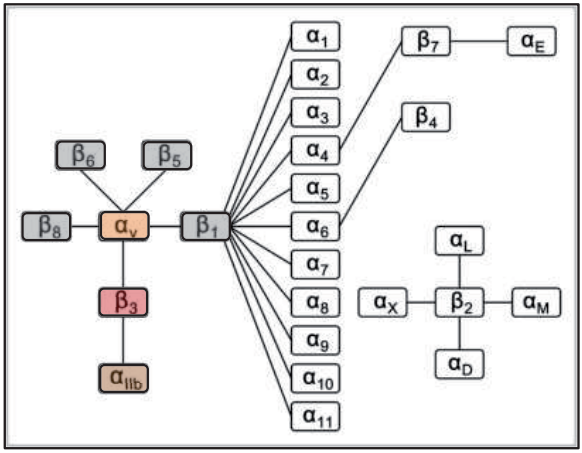

Figure 1: The integrin superfamily
The heterodimer formation of αv and αIIb with different β subunits is highlighted. Figure adapted from Chai and Chen, 2008.

The αvβ3 integrin, known as vitronectin receptor, represent as minor integrin on platelets (50-100 copies/platelet). However, αvβ3 integrin is found abundantly on osteoclasts, on endothelium and various other tissues such as, in tumors (Brooks et al. 1994; Horton 1997). Likewise, αvβ3 integrin plays an important regulatory role in angiogenesis (Mahabeleshwar et al. 2007; Weis & Cheresh 2011). In contrast to αIIb, αv can form heterodimers with different subunits (β1, β3, β5, β6, β8) (Figure 1). Interestingly, recent evidence showed the reciprocal involvement of αvβ3 and αvβ5 in angiogenesis, with αvβ3 play a role in modulating VEGF (Soldi et al. 1999; Mahabeleshwar et al. 2007). In addition, the ligation between αvβ3 and extracellular matrix is essential for cell survival. The inhibition of this interaction by monoclonal antibodies, RGD peptide or peptidomimetic agents will lead to cell apoptosis (anoikis) (Brassard et al. 2015; Eliceiri & Cheresh 1998; Brooks et al. 1994; Montgomery et al. 1994), and prevents angiogenesis (Montenegro et al. 2012; Maubant et al. 2006).

Recent structural analysis showed that both αIIb and αv consist of an amino-terminal β-propeller domain followed by a thigh domain and two calf domains. The β3 subunit has eight domains; an amino-terminal PSI (plextrin-

semaphorin-integrin) domain, Ig-like hybrid domain containing ligand-binding site, αA-hybrid domain, four EGF-like domains, and the β-tail domain. Under resting conditions, the headpiece of the integrin faces down towards the membrane and extends upwards in a "switchblade"-like opening upon activation (Figure 2). These long-range structural rearrangements of the entire integrin molecule involving inter-domain contacts appear closely linked to conformational changes within the I-like domains, which result in increased affinity and competence for ligand binding (Takagi & Springer 2002).

In humans, naturally occurring SNPs leading to single amino acid substitutions were found on αIIb and β3. By the identifications of the domains (see Figure 2), point mutations which are responsible for the formation of alloantigen determinant(s) can be located more precisely. Immunization against such polymorphic structure(s) expressed as human platelet antigens (HPA) may result in the production of platelet alloantibodies (see below).

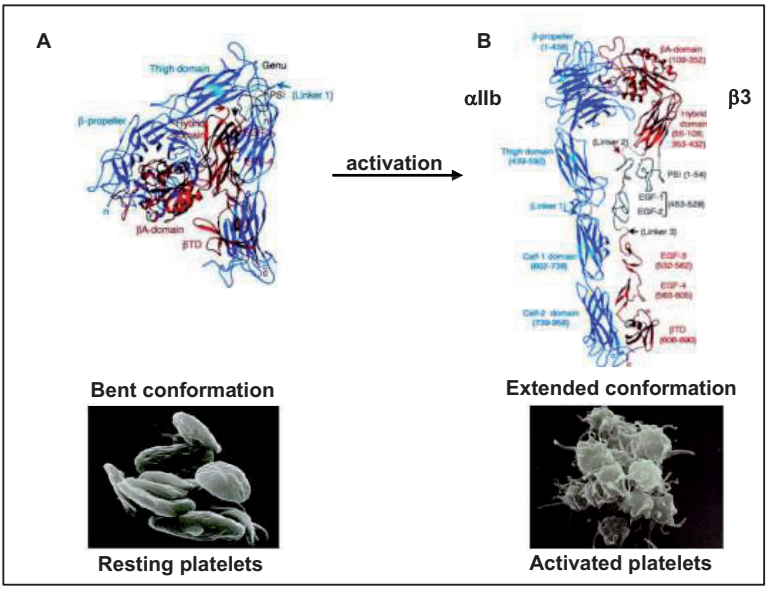

Figure 2: The three dimensional structure of integrin αIIbβ3
Schematic structure of the αIIbβ3 on resting platelets (A) and on activated platelets (B). Figure adapted from Xiong et al, 2001.

In some cases, SNPs can also impair αIIbβ3 synthesis leading to platelet dysfunction. Some SNPs on the αv subunit associated with susceptibility to chronic hepatitis B and hepatocellular carcinoma have been reported (Yang et al. 2014; Mathew et al. 2016). However, alloantibodies against αvβ3 have not been described so far.

1.2. SNPs on αIIbβ3 Integrin creating Alloantigenic Determinants

Integrin αIIbβ3 is the most immunogenic integrin on platelets (Table 1). Among common HPA systems, HPA-1 or previously designated as $PI^{A1/A2}$ or $Zw^{a/b}$ was the first HPA system implicated in FNAIT (van der Weerdt et al. 1963; Shulman 1962). HPA-1 system is a result of a single nucleotide change C>T that leads to an amino acid substitution leucine to proline at position 33. This point mutation is located on the PSI domain of the β3 subunit, a 13 amino acids long loop formed by disulfide bonds between cysteine residues 26 and 38 (Figure 3) (Newman et al. 1989; Calvete et al. 1991; Xiong et al. 2004). A rare mutation leucine into valine at position 33 has also been described (Santoso et al. 2006).

Table 1: Common Human Platelet Alloantigens (HPAs)

Antigen	Synonym	GP Location	Gene Name	Nucleotide Substitution	Aminoacid Substitution
HPA-1a	Zw^a, PI^{A1}	GPIIIa	ITGB3	T176	Leu33
HPA-1b	Zw^b, PI^{A2}			C176	Pro33
HPA-2a	Ko^b	GPIbα	GP1BA	C482	Thr145
HPA-2b	Ko^a, Sib^a			T482	Met145
HPA-3a	Bak^a, Lek^a	GPIIb	ITGA2B	T2621	Ile843
HPA-3b	Bak^b			G2621	Ser843
HPA-4a	Yuk^b, Pen^a	GPIIIa	ITGB3	G506	Arg143
HPA-4b	Yuk^a, Pen^b			A506	Gln143
HPA-5a	Br^b, Zav^b	GPIa	ITGA2	G1600	Glu505
HPA-5b	Br^a, Zav^a			A1600	Lys505

Although the location of amino acid substitution could be identified, little is currently known about the exact structure of HPA-1a alloantigenic determinant(s). Synthetic peptides could not mimic HPA-1a (Flug et al. 1991) indicating that the three dimensional structure of β3 integrin facilitated by multiple disulfide bonds, especially within the PSI domain, play an important role in the formation of HPA-

1a alloantigenic epitopes. Furthermore, some HPA-1a epitopes need additionally the presence of intact hybrid/EGF1 domains (Valentin et al. 2016). Naturally occurring substitution of the distally located residue 93 (Arg>Gln) at the hybrid/PSI interface disrupts the HPA-1a epitope for some anti-HPA-1a antibodies (Jallu et al. 2013).

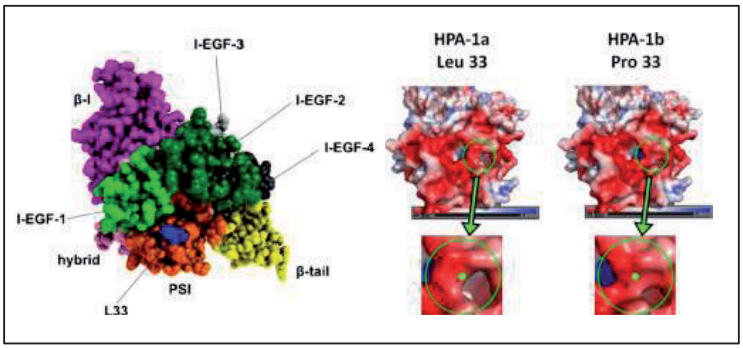

Figure 3: The ectodomain three-dimensional structure of the β3.
The PSI domain is harboring Leu33Pro mutation responsible for the formation of HPA-1a and HPA-1b, respectively. Figure adapted from Jallu et al. 2012

Additionally, the HPA-3 system located on the αIIb integrin subunit has been described (see Table 1). The HPA-3 system also known as $Bak^{a/b}$ results from a T>G base substitution at position 2621 located on the calf 2 domain of αIIb. This substitution determines the presence of isoleucine or serine at position 843 of the mature protein (Covas et al. 2000; Lyman et al. 1990). The HPA-3 alloantigen system is less well characterized, although it is known that anti-HPA-3 antibodies react with 76- and 60-Kd fragments of the GPIIb heavy chain. Unlike the HPA-1a system, the HPA-3 alloantigenic determinants do not seem to be sensitive to disulfide bond reduction, suggesting that they may comprise of the linear peptide (Landau & Rosenberg, 2011). However, Goldberger et al. (1991) and Calvete & Muniz-Diaz (1993) propose that the *O*-glycosylation site at serine 847 represents an additional critical component of HPA-3a epitopes. This carbohydrate residue seems to be a labile component; treatment of HPA-3a phenotyped platelets with neuraminidase reduced the binding affinity of some HPA-3a antibodies (Take et al. 1990). Besides common of HPAs, rare HPAs residing on αIIb and β3 subunits have been discovered. Currently, 13 HPAs

located on β3 subunit (see Table 2) and only a few rare HPAs were found on the αIIb subunit (see Table 3).

Table 2: Rare Human Platelet Alloantigens (HPAs) residing on β3

Antigen	GP Location	Gene Name	Nucleotide Substitution	Aminoacid Substitution	Reference
HPA-6b	GPIIIa	ITGB3	1544G>A	Arg489Gln	Wang et al. 1993
HPA-7b	GPIIIa	ITGB3	1297C>G	Pro407Ala	Kuijpers et al. 1993
HPA-7c	GPIIIa	ITGB3	1297C>T	Pro407Ser	Koh et al. 2010
HPA-8b	GPIIIa	ITGB3	1984C>G	Ag636Cys	Santoso et al. 1994
HPA-10b	GPIIIa	ITGB3	263G>A	Arg62Gln	Peyruchaud et al. 1997
HPA-11b	GPIIIa	ITGB3	1976G>A	Arg633His	Simsek et al. 1997
HPA-14b	GPIIIa	ITGB3	1909-1911 deletion AAG	Lys611del	Santoso et al. 2002
HPA-16b	GPIIIa	ITGB3	497C>T	Thr140Ile	Jallu et al. 2002
HPA-17b	GPIIIa	ITGB3	622C>T	Thr195Met	Stafford et al. 2008
HPA-19b	GPIIIa	ITGB3	487A>C	Lys137Gln	Peterson et al. 2010
HPA-21b	GPIIIa	ITGB3	1960G>A	Glu628Lys	Peterson et al. 2010
HPA-23b	GPIIIa	ITGB3	1942C>T	Arg622Trp	Peterson et al. 2012
HPA-26b	GPIIIa	ITGB3	1818G>T	Lys580Asn	Sachs et al. 2012
Kha[a]	GPIIIa	ITGB3	98C>T	Thr7Met	Sullivan et al. 2015

Table 3: Rare Human Platelet Alloantigens (HPAs) residing on αIIb

Antigen	GP Location	Gene Name	Nucleotide Substitution	Aminoacid Substitution	Reference
HPA-9b	GPIIb	ITGA2B	2602G>A	Val837Met	Noris et al. 1995
HPA-20b	GPIIb	ITGA2B	1949C>T	Thr619Met	Peterson et al. 2010
HPA-22b	GPIIb	ITGA2B	584A>C	Lys164Thr	Peterson et al. 2012
HPA-24b	GPIIb	ITGA2B	1508G>A	Ser472Asn	Jallu et al. 2011
HPA-27b	GPIIb	ITGA2B	2614C>A	Leu841Met	Jallu et al. 2013
HPA-28b	GPIIb	ITGA2B	2311C>G	Val740Leu	Poles et al. 2013
Lap[a]	GPIIb	ITGA2B	2511G>C	Gln806His	Wihadmadyatami et al. 2015

The fact that the polymorphic integrin β3 participates in the formation of the αvβ3 heterodimer is underestimated. Theoretically, this condition can also create single and/or complex antigenic epitopes which are recognized by the different types of platelet alloantibodies.

1.3 SNPs on β3 Integrin affecting Receptor Synthesis and Functions

Some SNPs on αIIb or β3 integrin can also affect mRNA stability and posttranslational processing of the proteins, and thereby impair the correct biosynthesis of αIIbβ3 (Bellucci & Caen 2002; Nurden 2006). In most cases, mutations result in low expression or total absent of αIIbβ3 expression on the platelet surface, which leads to functional defects of the receptor and, consequently, impaired platelet aggregation (hemostasis). Some of the mutations were found in the cytoplasmic domain of αIIb or β3 demonstrating the importance of this domain in integrin signaling (Chen et al. 1992; Peyruchaud et al. 1998; Raborn et al. 2011; Wang et al. 1997). In addition, others mutations located in the EGF domain of β3 give rise to a constitutively active receptor (Chen et al. 2001; Ruiz et al. 2001).

Interestingly, no correlation was observed between the amount of residual platelet αIIbβ3 and the severity of the hemorrhagic events (George et al. 1990; Nurden. 2006; Nurden et al. 2011). Since the β3 integrin subunit represents the common subunit of αIIbβ3 and αvβ3 heterodimer, a defect in β3 can theoretically alter the biogenesis and/or structure of αvβ3 integrin as well. It is feasible to speculate that absence of αvβ3 function in vascular cells contribute to hemorrhage (George et al. 1990; Nurden et al. 2011).

1.4. Immune Mediated Thrombocytopenia

In addition to their role in hemostasis, platelet αIIbβ3 integrin carries antigenic determinant(s), which are frequently targeted by platelet reactive antibodies recognizing either polymorphic structure (called alloantibodies) or common structure (called isoantibodies). Patients or mothers receiving "alloantigen-incompatible" platelets due to platelet transfusions and during pregnancy, respectively, usually produce platelet alloantibodies. Consequently, platelet reactive alloantibodies can cause alloimmune-mediated disorders, such as platelet transfusion refractoriness (PTR) and fetal neonatal alloimmune thrombocytopenia (FNAIT). Similarly, patients or mothers with β3 receptor deficiency (Glanzmann Thrombasthenia; see below) can produce platelet isoantibodies after platelet transfusion or pregnancy, respectively.

1.5. Diseases associated with platelet alloantibodies

The binding of platelet allo- or isoantibodies can induce platelet clearance by reticuloendothelial cells via Fcγ receptors. Three different disorders associated with this pathomechanism are known: Post Transfusion Purpura (PTP), Platelet Transfusion Refractoriness (PTR) and Fetal Neoanatal Alloimmune Thrombocytopenia (FNAIT).

1.5.1. Post Transfusion Purpura (PTP)

Post-transfusion purpura (PTP) is a rare transfusion reaction leading to severe thrombocytopenia which occurring approximately seven until ten days after blood transfusions. Its incidence is 1 in 50.000–100.0000 blood transfusions and occurs more commonly in multiparous women (Padhi et al. 2013). This disorder is mediated by alloantibodies against specific platelet antigens, most

commonly HPA-1a. However, the exact mechanism of PTP is unclear. Some studies have proposed that HPA-1a and HPA-1b share certain antigens, and thus could induce an alloantibody response, leading to platelet destruction. In addition was also proposed, the involvement of immune complexes (Schulman et al. 1961; Kaplan 2002). Intravenous immunoglobulin (IVIG) with and without corticosteroids has been used as first-line therapy (Kaplan 2002).

1.5.2. Platelet Transfusion Refractoriness (PTR)

PTR is defined as a failure of multiple platelet transfusions from random donors to improve the recipients platelet counts. Alloantibodies against human leucocyte alloantigen (HLA) class I as well as against HPAs have been documented (Kiefel et al. 2001). Transfusion of HLA and/or HPA compatible platelets is the treatment of choice (Hod and Schwartz,2008).

1.5.3. Fetal Neonatal Alloimmune Thrombocytopenia (FNAIT)

Fetal/neonatal alloimmune thrombocytopenia (FNAIT) is a bleeding disorder of the fetus and newborn caused by binding of maternal platelet alloantibodies to the infant's platelets, leading to their destruction during pregnancy or after birth (see Figure 1). Thrombocytopenia in the fetus/neonate is defined as platelet count $<150 \times 10^9$/L (Ghevaert, et al. 2007; Kjeldsen-Kragh et al. 2007). FNAIT is estimated to occurs in around 1:1,500-2,000 live births (Kjeldsen-Kragh et al. 2007; Kaplan 2006; Bussel et al. 2005) The occurrence of FNAIT in primiparous women is surprisingly high, more than 50% of all FNAIT cases. Antibodies against HPA-1a are the most frequent cause of FNAIT (80%) in Caucasians (Symington & Paes 2011). The clinical consequences of FNAIT include petechiae, hematoma, urogenital bleeding, intrauterine growth restriction, and fetal demise (Kaplan 2006).

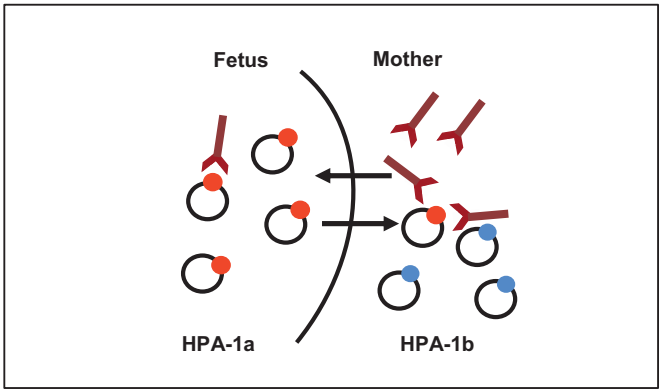

Figure 4: Mechanism of Fetal Neonatal Alloimmune Thrombocytopenia
During pregnancy, a HPA-1b positive mother (blue) develops anti-HPA-1a alloantibodies against HPA-1a positive platelets of the fetus (red). This IgG alloantibody can cross the placenta, binds to fetal platelets, leads to platelet destruction, and induces thereby thrombocytopenia and sometimes bleeding.

The most devastating risk of FNAIT is intracranial hemorrhage (ICH) leading to death or persistent neurological sequelae in approximately 10% of the clinically symptomatic cases (Figure 2) (Giers et al. 1996; Mueleler-Eckhardt et al. 1989.; Bussel et al. 2005; Spencer & Burrows 2001). It is currently unclear which factors determine whether ICH will occur. Modality of birth, birth weight, and the presence of other bleeding symptoms are not associated with ICH, and the association between low platelet counts and ICH is loose (Kroll et al. 2005; Thude et al. 2006). In subsequent pregnancies of mothers immunized against HPA-1a, only sibling history, but no laboratory test has been shown to be predictive for the risk of ICH (Birchall et al. 2003; Radder et al. 2003).

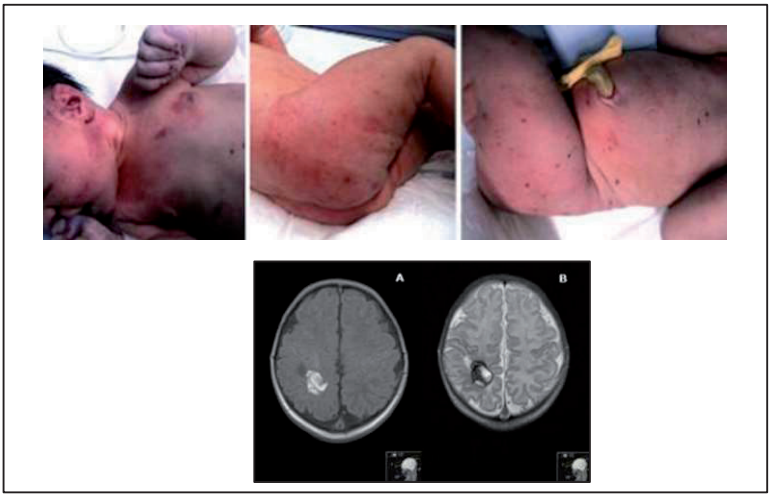

Figure 5: Clinical presentation of neonates with severe FNAIT
Generalized petechiae (upper) and cerebral bleeding (below) as shown by Magnetic Resonance Image. Figure adapted from Silva et al, 2011.

Routinely, the diagnosis of suspected FNAIT cases is approved by incompatibility between the HPA genotype of the mother and the child/father and by the identification of the corresponding platelet alloantibodies. To date, HPA typing is mainly performed by molecular-based methods (such as, PCR-SSP). Several methodological approaches for the detection of platelet alloantibodies exist. However, antigen capture assay (such as MAIPA; monoclonal antibody immobilization platelets specific alloantigens) is currently being used as the gold standard in platelet antibody detection (Kiefel et al. 1987).

In the prenatal period, treatment to maintain fetal platelets counts and to prevent bleeding during pregnancy are recommended including administration of intravenous IgG (IVIG) with and without corticosteroid in mothers, intrauterine transfusion with HPA-compatible platelets, fetal blood sampling (McQuilten et al. 2011), and fetal early delivery (Bussel 1997). Among these, IVIG seems to be most effective. It does not impair the maturation of the neonatal immune system (Rayment et al. 2011; Berkowitz et al. 2006).

Postnatal treatment aims to increase the platelets counts of the infant. Infusion of IVIG can be used although up to three days are required to reach a normal platelet count. Transfusion with HPA-matched platelets is recommended (McQuilten et al. 2011; Paterson et al. 2012). This approach shows higher platelet increment when compared to random platelets. Silent ICH in neonates with severe FNAIT has been observed (Bussel et al. 2005). Since the clinical diagnosis from ICH is not straight forward for all of neonates with suspected FNAIT, brain imaging is recommended especially for the neonates with the platelet count less than $50x10^9/L$.

1.6. Disease associated with platelet isoantibodies

Mutations based on genetic defects of αIIb or β3 are responsible for lacking, diminished expression, or functional defects of αIIbβ3 associated with a life long bleeding tendency, as observed in patients with Glanzmann Thrombasthenia. Transfusion of these patients with normal platelets from healthy donors can lead to the production of isoantibodies against αIIbβ3 integrin.

1.6.1. Glanzmann Thrombasthenia

Glanzmann Thrombasthenia (GT) is an inherited rare autosomal recessive bleeding disorder caused by a quantitative and/or qualitative deficiency of αIIbβ3. Laboratory analysis shows normal platelet count and morphology, but absent platelet aggregation responses to different platelet agonists (adenosine diphosphate, collagen, arachidonic acids, epinephrine) (Nair et al. 2016). However, platelets respond normally to ristocetin. GT patients are categorized into three types according to the number of αIIbβ3 expressed on the platelet surface. Type I GT patients express <5% αIIbβ3, type II GT patients express 5-20% αIIbβ3 and type III GT (variant) patients express a normal amount of αIIbβ3, but with functionally defects (Ambo et al. 1998; Siddiq et al. 2011). GT are found in several ethnic groups including Caucasians, Indians, Iranians, Iraqi Jews, Jordanian, Arabs and French Gypsies (Nurden et al. 2011; Chandrakala et al. 2012). Clinical symptoms of this disease occur early after birth and include episodic severe mucocutaneous or gastrointestinal bleeding, unprovoked bruising, epistaxis and prolonged bleeding after trauma or surgical interventions. In a woman, copious menstrual hemorrhage accompanies this disorder.

Recently, around 250 naturally occurring mutations in the αIIb (*ITGA2B*) or β3 (*ITGB3*) genes affecting biosynthesis and/or structure of the αIIbβ3 complex leading to GT disease have been reported; 138 mutations on ITGA2B (138 mutations), and 101 mutations on ITGB3 (see http:// sinaicentral. mssm. edu/ intranet/ research/ glanzmann) (Fiore et al. 2012; Tokgoz, et. al., 2015).

Treatment of GT patients depends in disease severity. Mild bleeding is treated conventionally by compression, local haemostatic agents, hormonal therapy and anti-fibrinolytics. Treatment with platelet transfusion(s) can be necessary in more severe bleeding. It however, may provoke isoimmunisation against αIIbβ3, which in turn can lead to platelet transfusion refractoriness. To overcome this problem, treatment with recombinant factor VII, especially for refractory patients, is recommended (Poon et al. 2015). Mother with GT may develop isoantibodies directed against αIIbβ3 expressed of foetal platelets leading to the development FNAIT (Leticee et al. 2005; Siddiq et al. 2011).

1.7. Anti-HPA-1a alloantibodies may react with endothelial cells

Endothelial cells are the thin layer of cells that lines the basal surface of blood vessels. Therefore, alterations at the level of the endothelium are crucial to understand the nature of vascular diseases and organ damage (Hernandez et al. 2012). Naturally, the αvβ3 integrin is found on endothelial cells and plays an important role in angiogenesis and vasculogenesis during blood vessel development. Furthermore, upregulation of endothelial αvβ3 is observed during inflammation which is mediated by vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF).

Anti-endothelial antibodies were detected in tissues of kidney biopsies and sera of patients with rheumatic diseases and Systemic Lupus Erythematosus (SLE) (Lindqvist et al, 1971; Renaudineau et al. 1999). In addition, several investigations reported the presence of anti-endothelial antibodies in systematic autoimmune and vascular disorders such as Rheumatoid Arthritis, Wegener Granulomatosis (Sebastian et al, 2007), Kawasaki Disease (Grunebaum et al. 2002), Behcet's Disease (Cervera et al. 1994), Takayasus Arteritis, Microscopic Polyangitis (Chanseaud et al. 2003) and Inflammatory Bowel Diseases (Aldebert et al, 1995). Recently, our group could demonstrate that alloantibodies against

neutrophil antigen (HNA-3a) cross-react with endothelial cells, thereby interfere with endothelial function, leading to transfusion related acute lung injury (TRALI) (Bayat et al. 2015; Sachs et al. 2006). Furthermore, accumulated evidence shows that alloantibodies against HLA expressed on endothelial cells are responsible for organ transplant rejection (Valenzuela & Reed 2014).

Some studies investigating the effect of maternal anti-HPA-1a alloantibodies on the endothelial cells were performed in the past. Radder et al. (2004) described that anti-HPA-1a alloantibodies did not cause activation and damage of endothelial cells In contrast, Gils et al. (2009) found that HPA-1a alloantibodies could inhibit endothelial cell spreading and interfere with endothelial cell monolayer integrity. It has also been speculated that anti-HPA-1a antibodies may affect placental development (Tiller et al. 2012).

More recently, Yougbaré and coworkers (2015) demonstrated in an animal model that anti-β3 integrin antibodies can impair angiogenesis in the fetal brain, indicating that anti-HPA-1a antibodies reactive with endothelial cells may be responsible for the development of ICH. The question why and how only some anti-HPA-1a alloantibodies are capable of inducing ICH was the focus of our investigation.

2. The Objectives of the Study

This study arose from the following questions and considerations:

Although HPA-1a alloantibodies as a cause of FNAIT in Caucasian population are well established, little is currently known about the relevance of platelet antibodies in Southeast Asian population, especially in Malays (Malaysia and Indonesia). Consequently, analysis of platelet alloantibody specificities in suspected FNAIT among Malays is mandatory to approach this question (see manuscript # 1).

In Caucasians population, most of the FNAIT cases are provoked by platelet alloantibodies against HPA-1a. In some severe FNAIT cases, maternal anti-HPA-1a antibodies can induce intracranial hemorrhage in the fetus during the pregnancy, leading to disability or death. The mechanism how these antibodies can cause severe bleeding in the fetal brain is not well understood. Also, little is known about the prediction and specific treatment of this severe FNAIT. Several studies indicate that neither platelet counts nor platelet antibody titer seem to be reliable predictors for the occurrence of ICH. Recent data from our center and others suggest that endothelial disturbance caused by endothelial-reactive antibodies can trigger bleeding. In this study, the effects anti- HPA-1a antibodies on endothelial cells was investigated (see manuscript #2). Furthermore, studies on other related diseases should confirm the existence of such endothelial-reactive antibody (see manuscript #3).

More recently, our group has demonstrated that a de-glycosylated mouse monoclonal IgG antibody against HPA-1a (called SZ21) can prevent platelet destruction and can be transported through the placenta making (rendering) prenatal treatment of FNAIT feasible. Meanwhile, this monoclonal antibody is humanized (called 813) and can be produced as a recombinant protein in mammalian cells. We analyzed the effect of de-glycosylated 813 (called d-813) on endothelial function and asked the question whether d-813 could prevent endothelial dysfunction caused by maternal anti-HPA-1a antibodies (see manuscript # 4).

Besides HPA-1a, several rare HPAs associated with FNAIT have been discovered in the last two decades. Although most rare HPAs are found among Caucasian, some are detected exclusively in the Asian population. Hence, characterization of new platelet alloantigen is important to augment the role of platelet alloantibodies in the pathomechanism of FNAIT (see manuscript # 5).

3. Manuscripts

3.1. Manuscript #1

Platelets Reactive Alloantibody Responsible for Immune Thrombocytopenia in Malay Population

Mohamad Ismail Armawai, Afifah Hasan, Hevi Wihadmadyatami and Sentot Santoso.

Medical Journal of Indonesia, 2014; volume 23, pages 158 – 162; see attachment 1.

Abstract

Background: Alloantibodies against human platelet alloantigens (HPAs) are responsible for the development of platelet transfusion refractoriness (PTR) in patients receiving random platelets and bleeding disorder in babies with fetal neonatal alloimmune thrombocytopenia (FNAIT). Recently, our results based on the analysis of the allelic distribution of HPAs indicated that immunization may occur among Malay. In this study, we sought to analyze the frequencies of platelet reactive alloantibodies responsible for FNAIT and PTR in Malaysia.

Methods: Sera from suspected FNAIT (n = 295) and PTR (n = 74) were collected in five years period (2008- 2013) and tested for the presence of platelet reactive antibodies by the use of antigen capture assay.

Results: In 5/74 (5.41%) platelet specific antibodies against HPA-2b (n = 1), HPA-5a (n = 1), HPA-5b (n = 1), HPA-15b (n = 2) could be identified in our PTR cohort. In FNAIT cohort, platelet specific alloantibodies could be detected in 18 sera (6.10%) consisting anti-HPA-1a (n = 1), anti-HPA-3a (n = 3), anti-HPA-5a (n = 6), anti-HPA-5b (n = 6), anti-HPA-15a (n = 1), and anti-HPA-15b (n = 1).

Conclusion: Our study indicates that anti-HPA-3, -HPA- 5 and -HPA-15 antibodies seem to be the most platelet specific antibodies involved in FNAIT and PTR cases in Malaysian population. Since similar HPA allelic distribution among Malaysian and Indonesian populations have been observed, immunization against these three HPA systems are expected to be the most potential risk of alloimmune mediated platelet disorders in Indonesia.

3.2. Manuscript #2

Anti-endothelial αvβ3 Antibodies are a Major Cause of Intracranial Bleeding in Fetal/ Neonatal Alloimmune Thrombocytopenia.

Sentot Santoso, Hevi Wihadmadyatami, Tamam Bakchoul, Silke Werth, Nadia Al-Fakhri, Gregor Bein, Peter J. Newman, Jieqing Zhu, Volker Kiefel, Behnaz Bayat and Ulrich J. Sachs

Atherosclerosis, Thrombosis, and Vascular Biology, Thrombosis, and Vascular Biology, 2016, volume 36, pages 1517-24; see attachment 2.

Abstract

Objective - Fetal/neonatal alloimmune thrombocytopenia (FNAIT) is a severe bleeding disorder which can result in intracranial hemorrhage (ICH), leading to death or neurological sequelae. In Caucasians, maternal anti-HPA-1a antibodies (abs) are responsible for the majority of cases. No predictive factors for ICH are available to guide prophylactic treatment during pregnancy. In this study, we investigated abs from mothers with ICH-positive FNAIT and with ICH-negative FNAIT in order to identify serological and functional differences between the groups.

Approach and Results - In an antigen capture assay, we observed a stronger binding of +ICH abs to endothelial cell (EC)-derived αvβ3. By absorption experiments, we subsequently identified anti-HPA-1a abs of anti- αvβ3 specificity in the +ICH, but not in the -ICH cohort. Only the anti- αvβ3 subtype, but not the anti-β3 subtype, induced EC apoptosis of HPA-1a positive ECs by caspase-3/7 activation, mediated by reactive oxygen species. In addition, only the anti- αvβ3 subtype, but not the anti-β3 subtype, interfered with EC adhesion to vitronectin and with EC tube formation.

Conclusions - We conclude that the composition of the anti-HPA-1a antibody subtype(s) of themother may determine whether ICH occurs. Analysis of anti-HPA-1a abs of the anti- αvβ3 subtype in maternal serum has potential in the diagnostic prediction of ICH development and may allow for modification of prophylactic treatment in FNAIT.

3.3. Manuscript #3

Immunization Against αIIβ3 and αvβ3 in a Type 1 Variant of Glanzmann's Thrombasthenia caused by a Missense Mutation Gly540Asp on β3

Hevi Wihadmadyatami, Heike Berghofer, Lida Röder, Gregor Bein, Kathrin Heidinger, Ulrich J. Sachs and Sentot Santoso

Thrombosis and Haemostasis, 2016, volume 116, pages 262-71; see attachment 3.

Abstract

Treatment of bleeding in patients with Glanzmann's thrombasthenia (GT) can be hampered by iso-antibodies against the αIIbβ3 integrin, which cause rapid clearance of transfused donor platelets. Type 1 GT patients with a total absence of αIIbβ3 from the platelet surface are known to be susceptible to form such isoantibodies. In this study, we describe a type 1 GT patient with a missense mutation ($Gly_{540}Asn$) located in the EGF3 domain of the β3 integrin subunit. Cotransfection analysis in CHO cells demonstrates total absence of αIIbβ3 from the surface, based on inappropriate αIIb maturation. The patient's serum was reactive with αIIbβ3 and αvβ3 integrins in a capture assay, when platelets and endothelial cells were used. Two specificities could be isolated from the patient's serum, anti-αIIbβ3 and anti-αvβ3 isoantibodies. Both specificities did not interfere with platelet aggregation. In contrast, isoantibodies against αvβ3, but not against αIIbβ3, were able to disturb endothelial cell adhesion onto vitronectin, triggered endothelial cell apoptosis and interfered with endothelial tube formation. This intriguing finding may explain more recently observed features of fetal/neonatal iso-immune thrombocytopenia in children from type 1 GT mothers with intracranial haemorrhage, which could be related to anti-endothelial activity of the maternal antibodies.

In conclusion, we give evidence that two isoantibody entities exist in type 1 GT patients, which are unequivocally different, both in an immunological and functional sense. Further research on the clinical consequences of immunization against αvβ3 is required, predominantly in GT patients of childbearing age.

3.4. Manuscript #4

Human Monoclonal Antibody against HPA-1a inhibits anti-HPA-1a Mediated Endothelial Disturbance.

Hevi Wihadmadyatami, Tamam Bakchoul, Gregor Bein, Behnaz Bayat, Ulrich J. Sachs and Sentot Santoso

Manuscript in preparation; see attachment 4.

Abstract

In this study, we proved whether modified human monoclonal antibody against HPA-1a (mab 813), can prevent endothelial dysfunction induced by maternal anti-HPA-1a alloantibodies. For this purpose, we removed the N-linked carbohydrate component of mab 813 by digestion with N-glycanase. This modified mab 813 (termed d-813) has several advantages; can inhibit the binding of maternal anti-HPA-1a antibody, can pass placenta barrier, but cannot recognize by RES system. Thus, the use of such antibody for the prenatal treatment of FNAIT is feasible (Bakchoul et al. 2013). Here, we could demonstrate, the d-813 itself did not impair endothelial functions, but d-813 administration prevents endothelial dysfunction mediated by maternal anti-HPA-1a. In addition, our *in vivo* experiment using NOD/SCID mouse model showed that d-813 could also prevent platelets clearance induced by anti-HPA-1a antibodies. This work represents the first evidence for the use of modified mab as a drug for prenatal treatment of FNAIT.

3.5. Manuscript #5

Alloantibody against New Platelets Alloantigen (Lapa) on GPIIb is Responsible for a Case of Fetal/ Neonatal Alloimmune Thrombocytopenia.

Hevi Wihadmadyatami, Kathrin Heidinger, Lida Adriana Röder, Silke Werth, Martin Knorr, Gregor Bein, Ulrich J. Sachs and Sentot Santoso

Transfusion, 2015; volume 55, pages 2920 – 2929; see attachment 5.

Abstract

Background: Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is caused by the destruction of platelets (PLTs) in the fetus or newborn by maternal platelet antibodies that crossed the placenta during pregnancy.

Study design and method: In this study, we aim to elucidate the properties of a new PLT alloantigen (Lapa) that is associated with a severe case of FNAIT. Analysis of maternal serum with phenotyped PLTs by monoclonal antibody specific immobilization of platelet antigens showed positive reaction against PLT glycoprotein (GP) IIb/IIIa and HLA Class I expressed on paternal PLTs.

Results: In contrast to GPIIIa-reactive anti-HPA-1a, anti-Lapa alloantibodies precipitated predominantly GPIIb. Indeed, a point mutation G>C at position 2511located in Exon 25 of the ITGA2B gene was found in Lapa-positive donors. This mutation causes an amino exchange Gln>His at Position 806 located in the calf-2 domain of GPIIb. Lapa-positive individuals were not found in 300 random blood donors. Our expression study showed that anti-Lapa alloantibodies reacted with stable transfected HEK293 cells expressing the mutated GPIIb isoform (His806). CHO cells carrying this isoform, however, failed to react with anti-Lapa alloantibodies, indicating that Lapa epitopes depend on the Gln806His mutation and the carbohydrate composition of the GPIIb. This mutation did not hamper the binding of anti-HPA-3a, which recognizes a point mutation (Ile843Ser) located in calf-2 domain. Finally, we found that Lapa and some HPA-3a epitopes are sensitive to O-glycanase.

Conclusion: This study not only underlines the relevance of rare HPAs on the pathomechanism of FNAIT, but also helps to understand the pitfalls of serologic assays to detect anti-GPIIb alloantibodies.

4. Discussion

Immunization against platelet antigens are commonly occurring as a cause of immune mediated thrombocytopenia in Caucasians population, but little is known about the clinical relevance of platelet antibodies in Asian populations, especially in the multiethnic population living in Malaysia and Indonesia.

Here in the first paper, we described for the first time the role of platelet antibodies on the mechanism of alloimmune mediated thrombocytopenia in Malays. By analysis of two different patient cohorts (FNAIT and PTR), we found a high prevalence of platelet alloantibodies reactive against HPA-3a, HPA-5a, and HPA-5b in mothers with FNAIT. Interestingly, severe FNAIT cases associated with anti-HPA-3a antibodies were observed. In contrast, anti-HPA-5b and anti-HPA-15b alloantibodies were predominantly detected in the PTR cohort. Additionally, some PTR patients developed pan-reactive antibodies against αIIbβ3 and platelet GPIb/IX. Based on the similar HPA allelic distribution among Malaysian and Indonesian populations (Asmarinah et al. 2013; Armawai et al. 2015), immunization against HPA-3, -5 and -15 systems are expected to be the most critical risk for the development of FNAIT and PTR among both populations. In comparison to Caucasians population, immunization against HPA-1a is as a rare event (Armawai et al. 2015).

Based on these observations, screening of platelet antibodies against HPA-3, -5 and -15 systems should be recommended for the diagnosis of FNAIT and PTR. Transfusion with HPA compatible platelets should be considered to treat these patients adequately. Therefore, typing of blood donors for HPA and installation of a national donor registry for HPA typed platelets are obligatory to solve this problem in near future.

In Caucasians, anti-HPA-1a antibodies are the most relevant cause of FNAIT with ICH as the most serious complication, prenatally or postnatally. ICH can even occur in the fetus/neonate with normal platelet counts (Kroll et al. 2005; Bussel et al. 2010), indicating that low platelet counts alone are not sufficient to trigger the development of ICH. Other effects of anti-HPA-1a antibodies must be involved in the mechanism of ICH.

In the second paper, we found that anti-HPA-1a antibodies are heterogeneous, containing at least three different types of anti-HPA-1a alloantibodies that react with β3 alone, with αIIbβ3 (complex epitopes formed by αIIb and β3 subunits) or with αvβ3 (complex epitopes formed by αv and β3 subunits). Although the Leu33Pro mutation residing on the β3 integrin subunit is known to control HPA-1a epitopes (Newmann et al. 1989), the presence of other subunits (αIIb or αv) seems to be necessary for the formation of some antigenic determinant(s) (Valentin et al. 1995; Allen et al. 2012). Theoretically, all these epitopes can be recognized by the immune system. Indeed, by the use of an absorption approach using recombinant αIIbβ3 immobilized onto beads and β3-integrin transfected cells, we could identified these three types of anti-HPA-1a alloantibodies as interactive with αIIbβ3 complex (termed "type-P"; reactive with platelets only), β3 (termed "type-PE"; reactive with platelets and endothelial cells) and αvβ3 (termed "type-E"; reactive predominantly with endothelial cells). Interestingly, a high prevalence of type-E antibodies was detected in FNAIT mothers with babies with ICH. These results indicate that type-E antibodies may be directly responsible for the development of ICH, whereas the other two alloantibody types (type P and type PE) may promote platelet clearance (Figure 6).

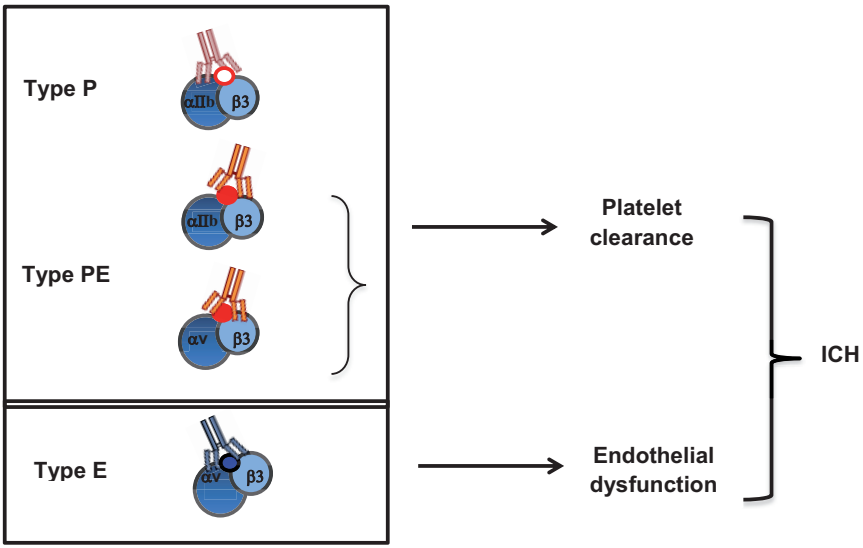

Figure 6: The three different subtypes of anti-HPA-1a alloantibodies
Antibodies type-P and type-PE are reactive against αIIbβ3 complex or β3 alone, respectively, and may induce platelet clearance, while type-E antibodies reactive with αvβ3 interfere with endothelial function. Both mechanisms can promote ICH, but the individual extent of each mechanism awaits clarifications.

It has been documented that αvβ3 is essential for endothelial proliferation and survival mediated by adhesion of endothelial cells onto extracellular matrix (Li et al. 1999). Inhibition of endothelial adhesion by αvβ3 antagonists (e.g. functional inhibitory monoclonal antibodies against αvβ3, cyclic RGD peptide, and peptidomimetics) that disrupt the ligation between αvβ3 and vitronectin lead to cell anoikis (Edreich and Epstein, 2005) and subsequently to cell apoptosis (Eliceiri & Cheresh 1999; Montgomery et al. 1994; Brassard et al. 2015; Brooks, 1994). During this process, cell detachment can also trigger mitochondria to produce reactive oxygen species (ROS). It is well known that ROS can initiate and regulate the transcription and activation of different mediators which culminate in the common mechanism of cell damage via caspase pathway (Elmore, 2007). Additionally, the αvβ3 integrin is expressed abundantly on activated endothelial cells during angiogenesis and plays a role as a key regulator of endothelial adhesion and migration (Somanath et al. 2009; Brooks, et

al. 1994; Galvagni et al. 2013). Vessel development in the brain depends on cross-talk between endothelial cells and perivascular astrocytes (Ballabh et al. 2004). For this process, αv integrins play an important role through the regulation of TGF-β activation and expression of TGF-β responsive genes that promote vessel differentiation and stabilization (Scaffidi et al. 2004; Wipff & Hinz 2008; Cambier et al. 2005).

With respect to this pathomechanism, we found (manuscript #2) that anti-HPA-1a antibodies against endothelial αvβ3 (type-E) prevent endothelial adhesion, leading to anoikis, apoptosis of endothelial cells and disturbed vessel development during angiogenesis in a ROS dependent manner (Figure 6). This phenomenon could not be observed with anti-HPA-1a antibodies of type-P and type-PE. Thus, this antibody-mediated mechanism of endothelial dysfunction may explain brain hemorrhage in FNAIT. These findings provide new insights into the mechanism of severe FNAIT associated with intracranial hemorrhage and open the way for the development of new diagnostic method and treatment strategy to prevent ICH.

Interestingly, same evidence was found in a patient with type 1 Glanzmann's thrombasthenia (GT) (manuscript #3) who ultimately failed to express αIIbβ3 and αvβ3 on his platelets surface due to a novel point mutation Gly540Asp located on the β3 integrin subunit. By transfection analysis, we found that this point mutation was directly responsible for the absence of both integrins. The amino acid glycine at position 540 is highly conserved across species and is located within a structurally important EGF3 domain of the β3 chain surrounded by three disulfide bonds, Cys536-544, Cys542-547 and Cys549-558 (Calvete & Muniz-Diaz 1993). It is well-known that type 1 GT patients with absent αIIbβ3 expression can develop isoantibodies against αIIbβ3, leading to platelet transfusion refractoriness (Poon et al, 2006). The question whether such patients can develop isoantibodies against αvβ3 was speculative. In this study, we could demonstrate that type 1 GT patients can also develop anti-αvβ3 isoantibodies.

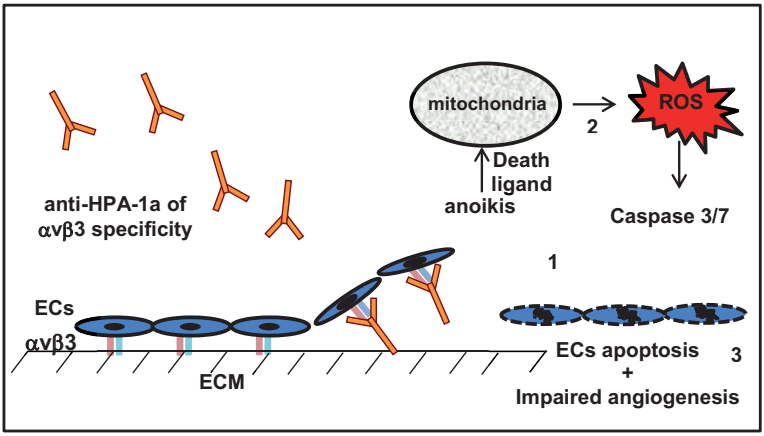

Figure 7: The possible mechanism of endothelial cell dysfunction mediated by anti-HPA-1a antibodies
1.Anti-HPA-1a antibodies bound to endothelial αvβ3 prevent adhesion of endothelial cells (ECs) onto extracellular matrix (ECM), leading to anoikis 2) Anoikis promotes apoptosis signaling via the external pathway (death ligand) and induces mitochondrial production of reactive oxygen species (ROS), and activation caspase 3/7 3) Caspase 3/7 triggers ECs apoptosis followed by disturbance of angiogenesis.

Furthermore, our functional analysis showed that this antibody specificity interfered significantly with endothelial functions (see manuscript #3). Whether these antibodies are able to contribute to platelet transfusion refractoriness remains unclear. However, the relevance of anti-αvβ3 isoantibodies need to be considered in pregnant GT women. These antibodies can pass the placenta and induce fetal/neonatal isoimmune thrombocytopenia, a condition similar to FNAIT (Sachs 2013). Recently, Siddiq et al. (2011) reported that maternal immunization against platelet antigens was found in a large cohort of GT mothers, and fetal or neonatal death occurred only in mothers with antibodies. Anti-αIIbβ3 isoantibodies were also identified in association with intracranial hemorrhage (ICH) in two FNAIT cases of GT type 1 mothers (Ambo et al. 1998; Leticee 2005). However, this αIIbβ3 antibody type was also found in healthy newborns without any signs of bleeding (Siddiq et al. 2011; Boval et al. 2001; Sundqvist et al. 1981; Ito et al. 1991).

Several efforts have been conducted to prevent severe thrombocytopenia and ICH. Currently, IVIG is widely used as an effective antenatal therapy for FNAIT and it prevents against ICH in most pregnancies (Bussel et al. 2010). More recently, Widiapraja and coworkers showed that IVIG may protect cells by reducing the inflammation rate (Widiapradja et al. 2014; Widiapradja et al. 2012).

It becomes increasingly clear that the Fc-linked glycosylation of IgG regulates the effector functions of IgG (Jefferis 2009). Deglycosylation of the N-glycan attached to Asn297 inhibits recognition by Fc receptors on macrophages (FcRI, FcRIIa, FcRIIIa) as well as its ability to activate complement factor C1q (Nimmerjahn & Ravetch 2008). Interestingly, FcRn-mediated IgG transport through the placenta does not require carbohydrate moieties on the Fc part (Palmeira et al. 2012; Einarsdottir et al. 2013). Thus, removal of the N-glycan should not affect placental transport.

Recent findings from our group demonstrated that deglycosylated mouse monoclonal anti HPA-1a antibodies (clone SZ21) could pass through the placenta and compete for the binding site of maternal HPA-1a alloantibody to prevent fetal platelet clearance by macrophages (Bakchoul et al. 2013). For the development of a drug, this monoclonal antibody was humanized (called now 813). To analyze the effect of 813 on endothelial function, deglycosylated 813 (d-813) was generated. Interestingly, this modified anti-HPA-1a antibody did not inhibit adhesion of endothelial cells onto vitronectin matrix, did not induce apoptosis and did not impair angiogenesis. When maternal anti-HPA-1a antibodies derived from ICH cases were tested, presence of d-813 antibodies prevented apoptosis and restored angiogenesis. Furthermore, our in-vivo analysis in NOD/SCID mice showed that d-813 antibodies could prevent platelet clearance mediated by maternal anti-HPA-1a antibodies. This observation indicates the therapeutic potential of d-813 for antenatal treatment of FNAIT, even with ICH. However, the precise mechanism how this modified monoclonal antibody can protect platelets and endothelial cells needs to be evaluated further.

In line with our findings, Eksteen et al. (2015) recently described a new human monoclonal antibody against HPA-1a (named 26.4) derived from a single memory B cell isolated from a woman who developed anti-HPA-1a antibodies.

This antibody inhibits the binding of maternal anti-HPA-1a antibodies, although the epitopes of 26.4 are not constrained to the PSI domain alone. This is in contrast to d-813 that reacts with small epitopes residing on the PSI domain (Stafford et al. 2008). Ghevaert et al. (2008) also proved that monoclonal antibody B2G1 derived from the maternal B cells of a FNAIT case by phage display could block the binding of maternal anti-HPA1a antibodies in vitro (Ghevaert, et al. 2007). The question which human HPA-1a antibody; 813, 26.4 or B2G1 is most effective in preventing platelet clearance and endothelial dysfunction caused by maternal anti-HPA1a antibodies is intriguing.

Administration of anti-integrin β3 sera prior to injection of integrin β3 positive platelets in an integrin β3-/- murine model of FNAIT prevented the formation of anti-platelet antibodies and reduced bleeding complications in newborn pups (Tiller et al. 2012). Currently, clinical trials for FNAIT prevention based on prophylaxis are underway to test the potential of anti-HPA-1a IgG to prevent HPA-1a immunization (Kjeldsen-Kragh et al. 2012). This project, however, needs a large number of women with anti-HPA-1a antibodies. Whether human monoclonal antibodies can replace this prophylaxis approach is an interesting question.

In recent years, a large number of low or rare frequency human platelets alloantigen has been discovered. Some of them are associated with severe FNAIT cases. On the last paper, we describe a new rare alloantigen term as Lap^a located on αIIb integrin subunit. Nucleotide sequencing of ITGA2B gene derived from the Lap^a-positive father showed one nucleotide substitution G>C at position 2511 located in exon 25 leading to amino acid substitution from glutamine to histidine at position 806 (Gln806His). In a population study, none from 300 unrelated donors was found to carry the Lap^a alloantigen. Transfection analysis mammalian cells confirmed that Gln806His mutation is directly responsible for the formation of Lap^a alloantigenic determinant. However, we found that glycosylation of αIIb was required for the appropriate Lap^a alloantigen. This observation is in line with previous studies demonstrating that HPA-3a located on αIIb subunit, determined by a single amino acid mutation (Ile843Ser) also depends on the O-glycosylation of Ser847 (Calvette et al. 1993; Djafar et al. 1993), and sialic acid residues (Goldberger et al. 1991; Wang et al. 2012). These

results indicate that two different types of anti-HPA-3a may develop during alloimmunization; type 1 recognizes carbohydrate-independent epitopes and type 2 reacts with carbohydrate-dependent epitopes. The contribution of carbohydrates on the formation HPAs residing on αIIb may help us to design better serologic assays to detect platelet reactive antibodies against αIIb.

5. Summary

Alloimmunization against HPAs leading to bleeding disorders is not only important for Caucasians but also Asians, especially in multiethnic Malays. This evidence promotes HPA typing and the installation of a national donor registry to improve the quality of treatment in Asia.

In Caucasians, the pathomechanism how intracranial bleeding caused by anti-HPA-1a occurs during pregnancy in FNAIT was unclear. In this study, we found a high prevalence of anti-HPA-1a antibodies reactive against endothelial $\alpha v\beta 3$ (type-E) in mothers with FNAIT. In vitro, we could demonstrate that this subtype impairs endothelial function, and interferes with angiogenesis, indicating that type-E antibodies are directly responsible for the development of ICH. Our study showed that type-E antibody can also be formed in type 1 GT patients receiving platelet transfusions. Similar to FNAIT, this antibody type impairs endothelial function as well as angiogenesis. Thus, the presence of type-E antibodies in pregnant GT mothers may also cause severe FNAIT. Our study with a modified monoclonal antibody, 813, showed that platelet clearance and endothelial dysfunction mediated by maternal anti-HPA-1a alloantibodies may be prevented by such an approach. This interesting approach may help us to prevent bleeding and ICH in the fetus during pregnancy.

In the last study, we showed that characterization of new platelet alloantigens involved in FNAIT does not only broaden our diagnostic repertoire but also improves our knowledge about the involvement of other molecule components (such as carbohydrates) on the formation of platelet alloantigenic determinants.

Altogether, this study does not only underline the importance of alloantibodies in platelet clearance, but also highlights the importance of endothelial-reactive alloantibodies in severe bleeding such as, ICH in FNAIT cases. This knowledge will promote further studies including the development of new diagnostic methods and new treatment strategies of alloimmune mediated disorders.

6. Zusammenfassung

Eine Alloimmunisierung gegen humane Thrombozytenantigene (Human Platelet Antigenes, HPAs) und dadurch bedingter hämorrhagischer Diathese findet sich gehäuft nicht nur bei Kaukasien, sondern auch bei Asiaten, insbesondere bei den multiethnischen malaiischen Völkern. Dieses erfordert eine HPA-Typisierung sowie eine nationale Spenderdatei, um die Qualität von Thrombozytentransfusionen in Asien zu verbessern.

Bei der bei Kaukasiern vorkommenden fetalen/neonatalen Thrombozytopenie (FNAIT) war bislang der Pathomechanismus der intrakranialen Blutungen ungeklärt, welche während der Schwangerschaft infolge vorhandener Anti-HPA-1a-Antikörper auftreten können. In einer eigenen Studie fanden wir bei Müttern, bei denen eine FNAIT diagnostiziert wurde, in hoher Prävalenz Anti-HPA-1a-Antikörper, welche mit dem endothelialem Integrin αVβ3 (Typ E) reagierten. In einem In-vitro-Modell konnten wir nachweisen, dass dieser Subtyp über eine Beeinflussung der Angiogenese die Endothelfunktion beeinträchtigen kann. Dies deutet darauf hin, dass solche Typ-E-Antikörper direkt für das Auftreten intrakranieller Blutungen (Intracranial Hemorrhage, ICH) verantwortlich sind. Unsere weiteren Untersuchungen zeigen ebenfalls, dass Typ-E-Antikörper auch von Glanzmann-Thrombasthenie-(GT)-Typ-1-Patienten nach Thrombozyten-Transfusionen gebildet werden können. Ähnlich wie bei der FNAIT beeinträchtigt dieser Antikörper sowohl die Endothelfunktion wie auch die Angiogenese. Daher kann das Vorhandensein von Typ-E-Antikörpern bei Schwangeren mit GT ebenfalls eine schwere FNAIT hervorrufen.
In einer andern Studie mit dem modifizierten humanen Antikörper 813 konnten wir nachweisen, dass die durch maternale Anti-HPA-1a-Alloantikörper vermittelte Thrombozyten-Clearance und die endotheliale Dysfunktion durch den modifizierten murinen monoklonalen Antikörper d-813 verhindert werden kann. Dieser interessante Ansatzpunkt könnte dazu beitragen, Blutungen und damit auch eine ICH beim Fetus während der Schwangerschaft zu verhindern.

In unseren letzten Studie haben wir gezeigt, dass die Charakterisierung neuer, im Zusammenhang mit der FNAIT stehender Thrombozytenantigene nicht nur unsere diagnostischen Möglichkeiten bezüglich dieser Krankheit erweitert,

sondern auch unsere Kenntnisse über die Beteiligung anderer Moleküle (wie etwa Kohlenhydrate) bei der Bildung thrombozytärer Alloantigene.

Insgesamt demonstrieren die Ergebnisse dieser Studien nicht nur die Bedeutung von Alloantikörpern bei der Thrombozyten-Clearance, sondern sie unterstreichen auch die Bedeutung Endothel-reaktiver Alloantikörper beim Auftreten schwerer Blutungen, wie etwa bei der ICH in Fällen von FNAIT. Diese Kenntnisse können als Grundlage dienen für weitere Forschungen in Hinblick auf die Entwicklung neuer diagnostischer Methoden und neuer Behandlungsstrategien bei Alloimmunkrankheiten.

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8. Erklärung

Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichen oder nicht veröffentlichen Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der ,,Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis'' niederlegt sind, eingehalten.

Hevi Wihadmadyatami

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10. The First Publication (Attachment 1)

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Clinical Research

Platelet reactive alloantibodies responsible for immune thrombocytopenia in Malay population

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Abstrak

Latar belakang: *Aloantibodi terhadap* human platelet alloantigens *(HPAs) berperan dalam terbentuknya* platelet transfusion refractoriness *(PTR) pada pasien yang mendapat transfusi trombosit dan gangguan pembekuan darah pada bayi dengan trombositopenia aloimun fetalis (FNAIT). Hasil penelitian kami sebelumnya, berdasarkan analisis distribusi alel HPAs, mengindikasikan imunisasi mungkin terjadi antara orang Melayu. Pada penelitian ini, kami mencoba untuk menganalisis frekuensi aloantibodi reaktif trombosit yang berperan dalam FNAIT dan PTR di Malaysia.*

Metode: *Serum dari pasien diduga FNAIT (n = 295) dan PTR (n = 74) dikumpulkan selama lima tahun (2008-2013) dan diuji untuk mengetahui adanya antibodi platelet reaktif dengan pemeriksaan penangkapan antigen.*

Hasil: *Pada 5/74 (5,41%), antibodi platelet spesifik terhadap HPA-2b (n = 1), HPA-5a (n = 1), HPA-5b (n = 1), HPA-15b (n = 2) dapat diidentifikasi dalam kelompok kohort PTR. Dalam kohort FNAIT, aloanthodi spesifik trombosit dapat dideteksi pada 18 serum (6,10%) yang terdiri atas anti-HPA-1a (n = 1), anti-HPA-3a (n = 3), anti-HPA-5a (n = 6) , anti-HPA-5b (n = 6), anti-HPA-15a (n = 1), dan anti-HPA-15b (n = 1).*

Kesimpulan: *Penelitian ini memperlihatkan bahwa antibodi anti-HPA-3, -HPA-5 dan -HPA-15 merupakan antibodi spesifik trombosit yang terbanyak dalam kasus FNAIT dan PTR di populasi Malaysia. Karena distribusi alel HPA yang sama terjadi antara populasi Malaysia dan Indonesia, imunisasi terhadap tiga sistem HPA ini diperkirakan menjadi risiko paling potensial untuk gangguan trombosit yang dimediasi aloimun di Indonesia.*

Abstract

Background: Alloantibodies against human platelet alloantigens (HPAs) are responsible for the development of platelet transfusion refractoriness (PTR) in patients receiving random platelets and bleeding disorder in babies with fetal neonatal alloimmune thrombocytopenia (FNAIT). Recently, our results based on the analysis of the allelic distribution of HPAs indicated that immunization may occur among Malay. In this study, we sought to analyze the frequencies of platelet reactive alloantibodies responsible for FNAIT and PTR in Malaysia.

Methods: Sera from suspected FNAIT (n = 295) and PTR (n = 74) were collected in five years period (2008-2013) and tested for the presence of platelet reactive antibodies by the use of antigen capture assay.

Results: In 5/74 (5.41%) platelet specific antibodies against HPA-2b (n = 1), HPA-5a (n = 1), HPA-5b (n = 1), HPA-15b (n = 2) could be identified in our PTR cohort. In FNAIT cohort, platelet specific alloantibodies could be detected in 18 sera (6.10%) consisting anti-HPA-1a (n = 1), anti-HPA-3a (n = 3), anti-HPA-5a (n = 6), anti-HPA-5b (n = 6), anti-HPA-15a (n = 1), and anti-HPA-15b (n = 1).

Conclusion: Our study indicates that anti-HPA-3, -HPA-5 and -HPA-15 antibodies seems to be the most platelet specific antibodies involved in FNAIT and PTR cases in Malaysian population. Since similar HPA allelic distribution among Malaysian and Indonesian populations have been observed, immunization against these three HPA systems are expected to be the most potential risk of alloimmune mediated platelet disorders in Indonesia.

Keywords: alloimmune thrombocytopenia, transfusion

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Human platelet alloantigens (HPAs) are immunogenic polymorphic forms of platelet membrane glycoproteins (GPs) GPIa, GPIb, GPIIb, GPIIIa and CD109. Meanwhile, 28 HPA biallelic systems have been discovered, all of which except one are defined by single amino substitution, caused by single nucleotide polymorphism (SNP) in the respective gene;[1] (see official homepage http://www.ebi.ac.uk/ipd/hpa/for recent update).

Alloantibodies against HPAs are involved in three major clinical syndromes: fetal neonatal alloimmune thrombocytopenia (FNAIT), platelet transfusion refractoriness (PTR), and post transfusion refractoriness (PTP). In addition, recent evidence indicated that antibodies against HPAs are also importance in transplantation settings.[2]

FNAIT is caused by HPA incompatibility between mother and fetus. During the pregnancy, maternal alloantibodies cross the placenta and mediate the destruction of fetal platelets. In Caucasian, anti-HPA-1a is the most commonly involved in FNAIT (75%), followed by anti-HPA-5b antibodies.[3] In PTR cases, patients fail to respond adequately to an appropriate dose of platelet transfusion. The immune-related PTR is mostly caused by antibodies against HLA class I antigens. However, alloantibodies against HPAs have been observed in 20-30% of PTR cases, mostly against HPA-1b and HPA-5b.[4] PTP is a rare transfusion reaction characterized by sudden onset of thrombocytopenia. The antibodies (mostly anti-HPA-1a) present in the recipient cause not only the destruction of donor's platelets but also autologous platelets. The mechanism underlying this phenomenon, however, is still unclear.[5]

Presently, the diagnosis of alloimmune mediated thrombocytopenia relies on the assignment of HPA incompatibility and on the identification of anti-HPA alloantibodies. Based on the underlying SNPs, several molecular biological methods have been established in the last decade, which now allow reliable high-resolution and high-throughout genotyping analysis of HPAs.[6] On account of these technologies, extensive studies on the gene frequency of HPAs have been conducted worldwide. However, the identification of antibodies is mandatory to define the correct clinical diagnostic and treatment strategy of patients suffering from alloimmune thrombocytopenia.

Recently, the distribution of HPA gene frequencies has been reported in Malaysia as well as Indonesia.[7,8] Until today, however, no data is available about the frequencies and the clinical relevance of HPA alloantibodies in these countries. In this study, we presented the first data according the frequencies of platelet reactive alloantibodies in Malaysia, which are responsible for FNAIT and PTR.

METHODS

Patients

Sera from suspected FNAIT (n = 295) and PTR (n = 74) were collected in five years period (2008-2013). Incompatibility on HPA systems was verified by genotyping of maternal/paternal or donor/recipient DNA. Genomic DNA was isolated from the leukocytes samples, and then genotyped by the allele-specific PCR as recommended by the manufactures (GTI, San Diego, CA, USA and BAGene, Lich, Germany). PCR products were analyzed by 2% agarose gel electrophoresis and were visualized using a UV transilluminator. Informed consent was obtained from all subjects and the study was approved by the institution's review board.

Monoclonal antibodies (Mabs)

Mabs against glycoprotein (GP) IIb/IIIa (clone Y2/51; DakoCytomation; Denmark), GPIb/IX (clone SZ2; Beckman Coulter, Marseille, France), GPIa/IIa (clone Gi9; Beckman Coulter, Marseille, France), CD109 (clone TEA/16; BD Pharmingen, USA), and specific for HLA Class I (clone B.9.12.1) were used for the antigen capture assay (see below).

Detection of platelet alloantibodies by antigen capture assay

Alloantibodies were analyzed using a panel of known HPAs typed platelets (HPA-1,-2,-3,-5, and -15) from blood group O and paternal platelets by antigen capture assay, MAIPA (monoclonal antibody immobilized platelet antigen) as previously described.[9,10] The principle of this assay is illustrated in figure 1.

In brief, $100x10^9$/L washed platelets of HPA phenotyped donors were incubated with 25µL of human serum for 30 min at 37°C. After washings with 200 µL TBS/BSA buffer (0.099M Tris buffered saline containing 0.2% bovine serum albumin), sensitized platelets were incubated with mabs for 30 min at 37°C and washed again 3 times with TBS/

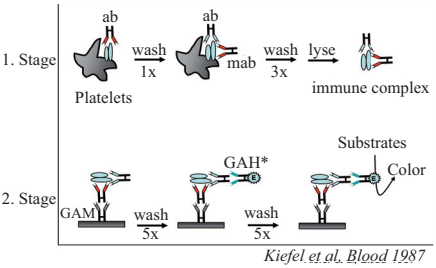

Figure 1. The priciple of the MAIPA assay

BSA buffer. Subsequently, platelets were solubilized for 15 min at room temperature (RT) with 130µL solubilization buffer (TBS containing 0.5% Triton X-100) prior to centrifugation for 15 min at RT (13,000 g). The supernatants were then transferred to an F-microtiter plate coated with goat anti-mouse IgG (dilution 1:500; Jackson Immunoresearch, Pennsylvania, USA). After incubation (30 min at 37°C) the wells are tipped out and washed 5 times with 125µL washing buffer (0.099M TBS containing 0.5% NP40 and 0.2% Tween 20), and incubated with 100µL of peroxidase conjugated goat anti-human IgG (Jackson Immunoresearch; dilution 1/6000) for 1 h at RT. Finally, the wells were washed 5 times with 125µL washing buffer. The reaction was visualized with 100uL of ortho-pheneylendiamin (Dako) substrate solution (15 min in the dark at RT), stopped by adding 100µL 0.5M H_2SO_4, and measured in photometer at 490nm (Dynex Technologies Inc., Virginia, USA).

RESULTS

In total of 295 samples of suspected FNAIT cases, platelet antibodies against HPAs were found in 18 sera (6.10%) by the MAIPA which consist of alloantibodies against HPA-1a (n = 1), HPA-3a (n = 3), HPA-5a (n = 6), HPA-5b (n = 6), HPA-15a (n = 1), and HPA-15b (n = 1). In addition, platelet reactive antibodies against HLA class I molecule could be detected in 58 samples (19.67%) (Table 1). Current evidence indicate that anti-HLA class I antibodies do not seem to play a major role on the mechanism of FNAIT. However, limited cases of FNAIT associated with HLA class I antibodies have been described.[11] In PTR cases, platelet specific antibodies against HPAs were detected in 4/74 cases only (5%). Anti-HPA-2b (n = 1), anti-HPA-5a (n = 1), anti-HPA-5b (n = 2), anti-HPA-15b (n = 3) could also be identified. In addition, we also found pan-reactive antibodies against GPIIb/IIIa (n = 11) and GPIb/IX (n = 1) in 16.2% of PTR cases.

Table 2 shows the allelic distribution of HPA systems in Malaysia and Indonesia. No significant different between these two populations could be observed. The HPA-1b allele frequency among Malay (both in Malaysia and Indonesia) was significantly lower than the frequency reported among Caucasian population (0.024 versus ~0.133). Thus, in contrast to Caucasian, alloimmune mediated thrombocytopenia caused by anti-HPA-1a antibodies seems to be extremely rare occurrence among Malay ethnics. However, we found a case of FNAIT associated with anti-HPA-1a antibodies. Furthermore, different allelic

Table 1. The frequencies of platelet antibodies found in suspected FNAIT and PTR cases

Alloantibody specificity	FNAIT (n = 295)	PTR (n = 74)
HLA class I	58	24
HPA-1a	1	-
HPA-2b	-	1
HPA-3a	3	-
HPA-5a	6	1
HPA-5b	6	1
HPA-15a	1	-
HPA-15b	1	2
GPIIb/IIIa	-	11
GPIb/IX	-	1

Table 2. Gene frequencies of HPA-1 to HPA-5 and HPA-15 in Malaysia (n = 200) and Indonesia. (n = 500) (Tan et al, 2012; Asmarinah et al 2013)

HPA system	Gene frequency Malaysia	Gene frequency Indonesia	Gene frequency Caucasian
HPA-1a	0.975	0.977	0.877
HPA-1b	0.025	0.023	0.133
HPA-2a	0.962	0.940	0.943
HPA-2b	0.038	0.060	0.057
HPA-3a	0.502	0.507	0.471
HPA-3b	0.498	0.493	0.529
HPA-4a	0.995	0.948	1.000
HPA-4b	0.005	0.052	0.000
HPA-5a	0.950	0.968	0.929
HPA-5b	0.050	0.032	0.071
HPA-15a	0.515	0.551	0.495
HPA-15b	0.485	0.449	0.505

distribution was observed for HPA-3 and HPA-15 systems. In Malays, HPA-3a and HPA-15a represent the frequent allele whereas in Caucasian population is contrariwise. Since HPA-3a/HPA-3b and HPA-15a/HPA-15b alleles are equally distributed, alloimmunization against these HPA systems is expected.

DISCUSSION

This study was aimed to evaluate the clinical impact of platelet reactive alloantibodies responsible for the pathomechanism of FNAIT and PTR cases in Malaysia. High prevalence of anti-HPA-3a, anti-HPA-5a, and anti-HPA-5b alloantibodies was found in our FNAIT cases. In this cohort, anti-HPA-3a alloantibodies were associated with severe thrombocytopenia (platelet count < $50x10^9$/L) in the newborns. This is in accordance with the current data reported by the Japanese Society on Platelet Serology.[12] All of the neonates was admitted to the NICU (Neonatal Intensive Care Unit) for close monitoring and received intravenous gamma-globulin (IVIG; 0.4g/kg body weight over five days) as recommended.[13] Based on the delay of IVIG response (24-48 hours), neonates were transfused with mother's platelets (HPA-3a negative) in order to maintain thrombocytopenia and bleeding tendency in these babies. Unfortunately, more improved therapy regiment by transfusion with HPA-3 negative platelets from healthy individuals was not possible due to a limited register of HPA typed donors in our National Blood Centre in Kuala Lumpur. To prevent long-term disability or death of neonates caused by intracranial hemorrhage during the pregnancy in suspected severe FNAIT cases, early identification of anti-HPA-3a alloantibodies is recommended.[14] In accordance to previous observation, only mild thrombocytopenia was observed in all FNAIT cases associated with anti-HPA-5a and -5b.[15]

In our PTP cohort, we found high prevalence of anti-HPA-5b and anti-HPA-15b alloantibodies, and quite high number of pan-reactive antibodies (without allele specificity) against platelet GPIIb/IIIa and GPIb/IX. The question whether this pan-reactive antibodies represents autoantibodies are difficult to answer, because we are unable to perform direct antibody testing on patient's platelet due to limited amount of platelets.

Based on the similar HPA allelic distribution among Malaysian and Indonesian population, immunization against HPA-3, -5 and -15 systems are expected to be the most potential risk for the development of FNAIT and PTR among Indonesian population. Therefore, screening of platelet antibodies against these three HPA systems should be recommended for the diagnosis of FNAIT and PTR. In antibody positive patients, HPA-compatible platelet transfusion should be given to improve the treatment of these disorders. Beside HPA-compatible platelets, however, compatibility between HLA antigens should also take into account when anti-HLA class I antibodies are also found. Providing HPA and HLA compatible platelets for patient's in-need is challenging task. Therefore, typing of many donors for these antigen systems and performing national donor registry is mandatory to solve this problem.

In conclusion, our study indicates that anti-HPA-3, -5 and -15 antibodies seems to be the most clinically important platelet specific antibodies involved in FNAIT and PTR cases in Malaysian population. Because of the lability of the HPA-3 and HPA-15 antigenic structures located on GPIIb and CD109, respectively, the detection of anti-HPA-3 and anti-HPA-15 alloantibodies is challenging and therefore some of the cases may overlook by the current assays. The use of fresh platelets is recommended to enhance the sensitivity of platelet antibody detection.[16-18]

Conflict of interest

All authors have nothing to disclose.

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11. The Second Publication (Attachment 2)

Antiendothelial αvβ3 Antibodies Are a Major Cause of Intracranial Bleeding in Fetal/Neonatal Alloimmune Thrombocytopenia

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Objective—Fetal/neonatal alloimmune thrombocytopenia is a severe bleeding disorder, which can result in intracranial hemorrhage (ICH), leading to death or neurological sequelae. In whites, maternal anti–human platelet antigen-1a (HPA-1a) antibodies are responsible for the majority of cases. No predictive factors for ICH are available to guide prophylactic treatment during pregnancy. In this study, we investigated antibodies from mothers with ICH-positive fetal/neonatal alloimmune thrombocytopenia and with ICH-negative fetal/neonatal alloimmune thrombocytopenia to identify serological and functional differences between the groups.

Approach and Results—In an antigen capture assay, we observed a stronger binding of +ICH antibodies to endothelial cell (EC)–derived αvβ3. By absorption experiments, we subsequently identified anti–HPA-1a antibodies of anti-αvβ3 specificity in the +ICH but not in the −ICH cohort. Only the anti–αvβ3 subtype, but not the anti–β3 subtype, induced EC apoptosis of HPA-1a–positive ECs by caspase-3/7 activation, and mediated by reactive oxygen species. In addition, only the anti–αvβ3 subtype, but not the anti-β3 subtype, interfered with EC adhesion to vitronectin and with EC tube formation.

Conclusions—We conclude that the composition of the anti–HPA-1a antibody subtype(s) of the mother may determine whether ICH occurs. Analysis of anti–HPA-1a antibodies of the anti–αvβ3 subtype in maternal serum has potential in the diagnostic prediction of ICH development and may allow for modification of prophylactic treatment in fetal/neonatal alloimmune thrombocytopenia. (***Arterioscler Thromb Vasc Biol*****. 2016;36:1517-1524. DOI: 10.1161/ATVBAHA.116.307281.)**

Key Words: antibodies ■ endothelial cells ■ intracranial hemorrhages ■ reactive oxygen species ■ thrombocytopenia, neonatal alloimmune

Fetal/neonatal alloimmune thrombocytopenia (FNAIT) is a bleeding disorder of the fetus and newborn in which maternal alloantibodies bind to the infant's platelets and cause their destruction during pregnancy and after birth. In whites, ≈80% of FNAIT cases are induced by antibodies against human platelet antigen 1a (HPA-1a).[1,2] The most devastating risk of FNAIT is intracranial hemorrhage (ICH), leading to death or persistent neurological sequelae in ≈10% of the clinically symptomatic cases.[3,4] It is currently unclear which factors determine whether ICH will occur. Modality of birth, birth weight, and the presence of other bleeding symptoms are not associated with ICH, and the association between low platelet counts and ICH is loose.[5,6] In subsequent pregnancies of mothers immunized against HPA-1a, only sibling history, but no laboratory test, has been shown to be predictive for the risk of ICH.[7,8]

HPA-1a is formed by a single amino acid substitution ($Leu_{33}Pro$) located on the flexible PSI (plexins, semaphorins, and integrins) domain of the integrin β3 chain.[9] On platelets, the β3 chain forms heterodimers either with αIIb or with αv, which functions as a fibrinogen or vitronectin receptor, respectively.[10] In contrast to αIIbβ3, αvβ3 is also found on endothelial cells (ECs), smooth muscle cells, and different cultured cells.[11] Several studies demonstrated that HPA-1a is constitutively expressed on endothelial αvβ3.[12,13] It has also been shown that anti–HPA-1a antibodies can affect endothelial integrity and the spreading capability of these cells,[14] indicating that vascular damage may be involved in the pathomechanism of FNAIT. However, others have reported no effect of anti–HPA-1a antibodies on endothelial activation and integrity.[15] Recently, Yougbaré et al[16] demonstrated that anti–β3

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Nonstandard Abbreviations and Acronyms

EC	endothelial cells
FNAIT	fetal/neonatal alloimmune thrombocytopenia
HPA	human platelet alloantigen
ICH	intracranial hemorrhage
ROS	reactive oxygen species

isoantibodies produced in β3 knockout mice can induce ICH in pups by impairment of angiogenesis rather than by thrombocytopenia. The relevance of this finding for the development of ICH in humans is not clear, especially because ICH is far less frequent in humans than observed in the animal model, and a booster effect as described in mice is absent in men, that is, likelihood of ICH does not increase with the number of pregnancies in humans.[17] It seems likely that these differences are related to the fact that isoantibodies (in the murine model) and alloantibodies (in humans) are not alike. Furthermore, frequency and natural history of ICH in humans suggest that the composition of the maternal anti–HPA-1a antibody repertoire could diverge between FNAIT cases with and without ICH.

In this study, we asked the question whether a specific anti–HPA-1a antibody subtype exists in FNAIT cases with ICH, in comparison to FNAIT cases without ICH. Our results show that anti–HPA-1a antibodies in FNAIT cases with ICH bind specifically to the αvβ3 complex, trigger endothelial apoptosis via reactive oxygen species (ROS), and interfere with angiogenesis.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Anti–HPA-1a Antibodies From +ICH and –ICH Cases Show Different Binding Patterns

The binding of anti–HPA-1a antibodies derived from –ICH cases (n=18; Table 1) and +ICH cases (n=18; Table 2) to αIIbβ3 and αvβ3 integrins derived from HPA-1aa platelets and ECs was investigated in an antigen capture assay. As shown in Figure 1, no significant difference between the 2 cohorts was observed in their binding to platelet-derived αIIbβ3 (A). However, a significant difference was observed when binding to both platelet-derived αIIbβ3 and αvβ3 was analyzed (B). The differences between both cohorts became more significant when sera were tested against EC-derived αvβ3 immobilized by monoclonal antibodies against αvβ3 (C) and β3 (D). These results indicate that +ICH anti–HPA-1a might contain additional antibody specificity, most probably against αvβ3 compound epitope(s).

Anti–HPA-1a Antibodies From +ICH Cases Contain a Specific Anti–αvβ3 Subtype

To prove this hypothesis, we aimed to isolate anti-αvβ3 by absorbing +ICH sera with αIIbβ3-coated beads. For the evaluation of this approach, monoclonal antibodies against αIIbβ3,

Table 1. Clinical and Laboratory Details of –ICH Cases

	Mother			Newborn					
No.	Gravida/Para	Antibody Specificity	Week of Gestation	Sex	Platelet Nadir (G/L)	ICH	Other Bleeding Symptoms	Bleeding Excluded by	Anti-αvβ3 (OD)
1	II/I	HPA-1a	28+4	M	12	No	H	Ultrasound	0.300
2	II/II	HPA-1a	38+4	F	22	No	P, H	Ultrasound	0.033
3	NR	HPA-1a	36+5	M	29	No	P	Ultrasound	0.048
4	I/I	HPA-1a	40+0	M	6	No	P	Ultrasound	0.320
5	NR	HPA-1a	40+2	F	11	No	P	Ultrasound	0.030
6	NR	HPA-1a	28+0	F	24	No	P	Ultrasound	0.152
7	III/III	HPA-1a	36+5	F	13	No	P	Ultrasound	0.324
8	I/I	HPA-1a	41+0	F	5	No	P	Ultrasound	0.273
9	I/I	HPA-1a	38+0	M	15	No	P	Ultrasound	0.238
10	I/I	HPA-1a	41+2	M	24	No	P	Ultrasound	0.215
11	I/I	HPA-1a	38+2	M	15	No	P, H	Ultrasound	0.089
12	I/I	HPA-1a	40+0	F	3	No	P, H	Ultrasound	0.174
13	III/II	HPA-1a	37+5	F	13	No	P, H	Ultrasound	0.084
14	III/III	HPA-1a	39+3	F	18	No	H	Ultrasound	0.098
15	I/I	HPA-1a	38+4	M	13	No	P, H	Ultrasound	0.167
16	IV/II	HPA-1a	34+5	NR	7	No	P	Ultrasound	0.204
17	IV/III	HPA-1a	40+0	M	18	No	P	Ultrasound	0.289
18	II/II	HPA-1a	40+0	M	9	No	P, U	Ultrasound	0.245

F indicates female; H, hematoma; HPA-1a, human platelet antigen 1a; ICH, intracranial hemorrhage; M, male; NR, not reported; OD, optical density; P, petechiae; and U, hematuria.

Table 2. Clinical and Laboratory Details of +ICH Cases

No.	Mother			Newborn					Anti-αvβ3 (OD)
	Gravida/ Para	Antibody Specificity	Week of Gestation	Sex	Platelet Nadir (G/L)	ICH	Bleeding Localization	Other Bleeding Symptoms	
1	I/I	HPA-1a	38+0	F	18	Yes	Parenchymal, left frontoparietal lobe	No	0.674
2	I/I	HPA-1a	38+6	F	21	Yes	Parenchymal, left side	P, H	0.488
3	II/II	HPA-1a	40+3	M	3	Yes	NR	P, H	0.664
4	VI/V	HPA-1a	39+0	M	5	Yes	Parenchymal, left temporal lobe	P	0.800
5	II/II	HPA-1a	37+6	F	5	Yes	Subependymal, left side	P, H	0.777
6	II/I	HPA-1a	40+0	M	30	Yes	Left thalamus and internal capsule	P	0.402
7	I/I	HPA-1a	39+6	M	10	Yes	Periventricular, right side, and plexus cyst	P, H	0.999
8	IV/II	HPA-1a	34+4	NR	5	Yes	Intraventricular and periventricular		0.549
9	III/II	HPA-1a	36+6	F	45	Yes	Parenchymal, right temporal lobe, grade IV	P	0.685
10	III/III	HPA-1a	41+2	F	7	Yes	Parenchymal, left parietooccipital lobe	P, H	0.721
11	II/I	HPA-1a	36+0	F	14	Yes	Parenchymal, left parietotemporal lobe	P	0.689
12	II/I	HPA-1a	38+0	M	17	Yes	Parenchymal, right temporal lobe	H, U	0.780
13	II/II	HPA-1a	34+5	M	24	Yes	Intraventricular	P	0.548
14	NR	HPA-1a	NR	F	12	Yes	Parenchymal	No	0.369
15	III/I	HPA-1a	41+3	F	12	Yes	Parenchymal, left parietooccipital lobe and right frontal lobe	P, H	0.995
16	NR	HPA-1a	39+0	M	2	Yes	Parenchymal	P, H	0.565
17	I/I	HPA-1a	38+0	M	10	Yes	Porencephalic cyst, right frontal lobe	P, H	0.686
18	II/I	HPA-1a	NR	M	20	Yes	Parenchymal, left and right hemisphere	No	0.206

F indicates female; H, hematoma; HPA-1a, human platelet antigen 1a; ICH, intracranial hemorrhage; M, male; NR, not reported; OD, optical density; P, petechiae; and U, hematuria

β3, and αvβ3 complexes were first absorbed with these beads and tested with Chinese hamster ovary cells expressing either αIIbβ3 or αvβ3. Absorption removed completely anti-β3 and anti-αIIbβ3 but not anti-αvβ3 (Figure I in the online-only Data Supplement). Subsequently, all human sera were absorbed with αIIbβ3-coated beads, and the remaining anti–HPA-1a antibodies (absorbate) were retested by antigen capture assay (Figure 2A). Absorbates from both cohorts became largely nonreactive with platelet-derived αIIbβ3 and αvβ3, with no differences between the cohorts (upper panel). In contrast, only sera from +ICH, but not from -ICH cases, showed significant reaction with EC-derived αvβ3, confirming our assumption that +ICH sera contain anti–HPA-1a specifically reactive with αvβ3 complex. Note that absorption reduced overall reactivity for both cohorts significantly (in comparison to Figure 1), indicating that relevant amounts of anti-αIIbβ3 and anti-β3, present in sera from both cohorts, were removed by absorption.

To further confirm the presence of anti-αvβ3 in the +ICH cohort, anti–HPA-1a antibodies present in absorbates and antibodies eluted from αIIbβ3-coated beads (eluates) were investigated by immunoprecipitation using biotin-labeled αIIbβ3- or αvβ3-transfected Chinese hamster ovary cells (Figure 2B). In the absorbates, anti-αvβ3 reactivity could be detected in +ICH but not in –ICH cohort, and this antibody specificity did not show cross reactivity with αIIbβ3 (neither anti-αIIbβ3 nor anti-β3). In contrast, cross-reactive antibodies against αvβ3 and αIIbβ3 were found in eluates from both cohorts. All 18 immunoblots were evaluated by integrity density measurement, demonstrating that the difference between the +ICH cohort and the –ICH cohort for anti-αvβ3 was significant (P=0.00042) in the absorbate but nonsignificant for αvβ3 (P=0.13) and αIIbβ3 (P=0.48) in eluates. Taken together, our results suggest that 3 different subtypes of anti–HPA-1a antibodies can exist in FNAIT sera: anti-αIIbβ3, anti-β3, and anti-αvβ3. In contrast to –ICH cases, sera from +ICH cases contain significant amounts of the anti–αvβ3 subtype.

Anti–αvβ3 Subtype of Anti–HPA-1a Interferes With EC Functions

On the basis of the fact that the anti–αvβ3 subtype is present in sera from +ICH cases and reacts predominantly with ECs, we sought to investigate whether this antibody type interferes with EC function. All functional experiments were performed with IgG fractions obtained from human sera (n=9) from each cohort, both before (pre) and after (post) absorption with αIIbβ3-coated beads.

First, interference of receptor–ligand binding was analyzed by cell adhesion assay. ECs were incubated with isolated IgG fractions before adhesion onto vitronectin-coated wells. As shown in Figure 3A, +ICH IgG significantly inhibited EC adhesion (black), whereas –ICH IgG did not (white). Removal of anti–HPA-1a antibodies of the anti–β3 and

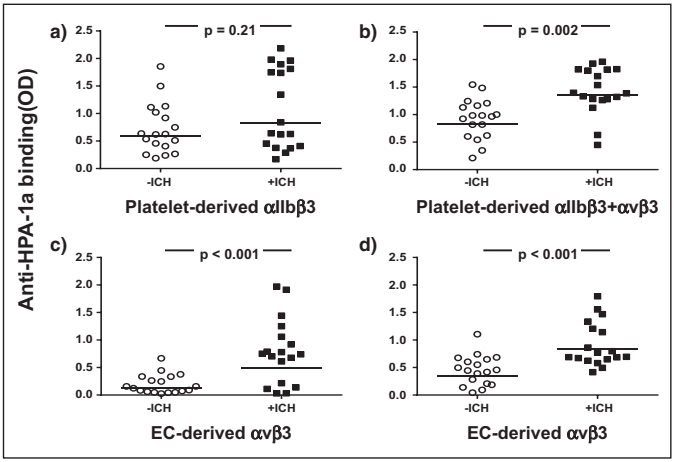

Figure 1. Analysis of maternal anti–human platelet antigen 1a (HPA-1a) derived from –intracranial hemorrhage (ICH; n=18) and +ICH (n=18) in an antigen capture assay. HPA-1aa platelets (**top**) or endothelial cells (**bottom**) were incubated with anti–HPA-1a sera and monoclonal antibodies (moabs) against αIIbβ3 (a), αvβ3 (c), and β3 (b and d). After cell lysis, the moab-(αIIbβ3/αvβ3/β3)–anti–HPA-1a trimolecular complex was immobilized on microtiter wells coated with antimouse IgG. Binding of anti–HPA-1a antibodies to platelet-derived αIIbβ3, αIIbβ3+αvβ3, and endothelial-derived αvβ3 was detected with enzyme-labeled anti–human IgG. Statistical analysis was performed by Mann–Whitney *U* test. EC indicates endothelial cell; and OD, optical density.

anti–αIIbβ3 subtypes (Figure 3A, post) had no influence on this effect, demonstrating that this effect was mediated by the anti–αvβ3 subtype.

It is known that disruption of EC adhesion onto extracellular matrix results in detachment-induced apoptosis, termed anoikis, which is associated with increased intracellular ROS level.[18,19] To analyze whether antibodies of the anti–αvβ3 subtype can also trigger endothelial anoikis, the generation of intracellular ROS induced by anti–HPA-1a antibodies was first measured by oxidation of DCFDA (2',7'-dichlorodihydrofluorescein diacetate) using flow cytometry. As shown in Figure 3B, only +ICH IgG (black) induced ROS, both pre and post absorption, indicating that the anti–αvβ3 subtype triggers ROS production. In the presence of AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride), a specific inhibitor of nicotinamide adenine dinucleotide phosphate oxidase,[20] ROS production was abrogated. Similar results were obtained with *N*-acetylcysteine (data not shown).

In accordance with these observations, binding of the anti–αvβ3 subtype triggered endothelial apoptosis as measured by caspase 3/7 assay (Figure 3C). In particular, the apoptosis rate induced by +ICH IgG remained unchanged after removal of the other subtypes (pre versus post; P=0.106). Apoptosis was confirmed by morphological assessment of chromatin DNA cleavage (Figure 3C).

Subsequently, an endothelial tube formation assay was performed to analyze whether the anti–αvβ3 antibody subtype would also affect angiogenesis (Figure 3D). +ICH IgG (black), but not –ICH IgG (white), significantly reduced tube length. Again, only the anti–αvβ3 subtype mediated the biological effect; removal of the other antibody subtypes by absorption had no influence on tube formation. The biological effect was ROS dependent and could be abrogated by AEBSF. Representative microphotographs are shown in Figure 3D (bottom). Finally, HPA-1bb ECs remained unaffected in all experiments in the presence of any of the IgG preparations (data not shown). Taken together, the anti–αvβ3 subtype bound specifically to HPA-1a expressed on ECs, inhibited cellular adhesion to vitronectin, caused cell apoptosis (anoikis), and consequently disturbed angiogenesis.

Detection of the Anti–αvβ3 Subtype as a Potential Predictive Parameter for ICH

Once anti–HPA-1a of the β3-subtype has been removed from the serum, clinically relevant anti–HPA-1a of the anti–αvβ3 subtype can be demonstrated in an antigen capture assay (Figure 2A, bottom). We noticed that the use of some monoclonal capture antibodies results in reduced sensitivity (Figure 2A, bottom, left), most probably caused by competitive inhibition between monoclonal and human antibodies. Adjusting the current assay (right diagram) to 100% specificity (no false-positive detection of anti–αvβ3 subtype in the –ICH cohort), 1 of 18 anti–HPA-1a of the anti–αvβ3 subtype cannot be identified in the +ICH cohort, resulting in a negative predictive value of 94.7%.

Discussion

The reason how and why ICH occurs in newborns with FNAIT is still unknown. The association between platelet count and ICH is weak, and ICH has been reported in FNAIT cases where the platelet count was within the reference range.[6,21] This indicates that biological effects of anti–HPA-1a antibodies other than increased platelet turnover may be responsible for the development of ICH.

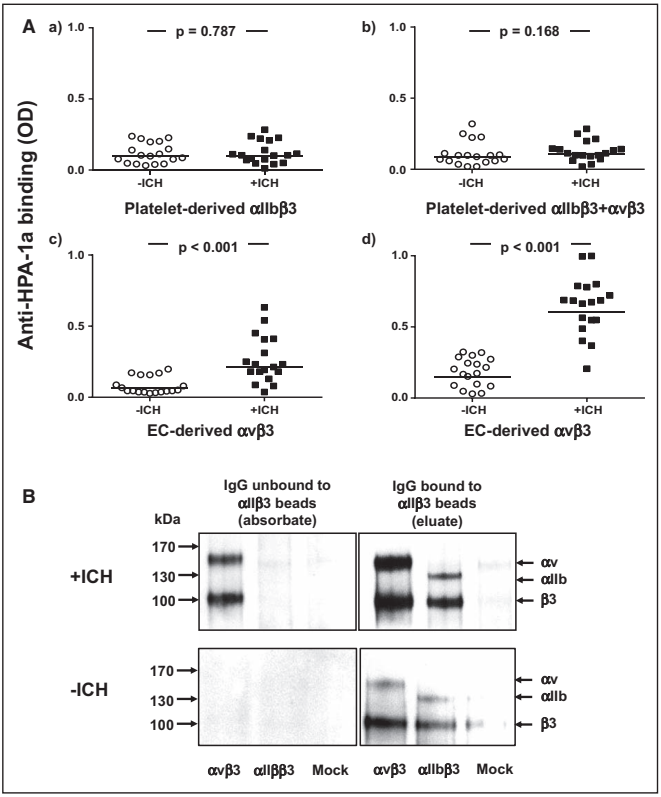

Figure 2. Identification of an anti–human platelet antigen 1a (HPA-1a) αvβ3-specific subtype in +intracranial hemorrhage (ICH) cases after absorption of other subtypes with αIIbβ3 beads. **A**, Maternal anti–HPA-1a antibodies (n=18 per cohort) were preabsorbed with αIIbβ3 beads to remove HPA-1a antibodies against αIIbβ3 and β3. Afterward, the absorbate was incubated with HPA-1aa platelets (**top**) or endothelial cells (ECs; **bottom**) and monoclonal antibodies (moabs) against αIIbβ3 (a), αvβ3 (c), or β3 (b and d). After cell lysis, the trimolecular antigen–antibody complex was immobilized on microtiter wells coated with antimouse IgG. Binding of anti–HPA–1a antibodies was detected with horseradish peroxidase–labeled anti–human IgG. Statistical analysis was performed by Mann–Whitney *U* test. Note that after absorption, αvβ3-specific anti–HPA-1a remains detectable in the +ICH cohort only (c and d). This antibody specificity reacts more readily with αvβ3 immobilized with moab AP3 (d) than with αvβ3 immobilized with moab 23C6 (c), possibly indicating epitope interference. **B**, Maternal anti–HPA-1a antibodies from the +ICH (**top**) and –ICH (**bottom**) cohorts were absorbed with αIIbβ3 beads. Unbound IgG (the absorbates) and bead-bound IgG (the eluates) were incubated with biotin-labeled transfected (αvβ3 and αIIbβ3) or nontransfected (mock) Chinese hamster ovary (CHO) cells, as indicated. After washings, CHO cells were lysed, and the antigen–antibody complex was precipitated with protein-G–coupled beads and separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis under nonreducing conditions. After blotting, precipitated proteins were visualized by the use of enzyme-labeled streptavidin and a chemiluminescence system. In the –ICH cohort (**bottom**), all antibodies were removed by the beads as indicated by a nonreactive absorbate (**left**). Antibodies of anti-αIIbβ3/anti-β3 specificity could be eluted from these beads (**right**). Note that anti-β3 is capable to pull down αIIbβ3 and αvβ3 integrins. In contrast, in the +ICH cases (**top**), removal of anti-αIIbβ3/anti-β3 antibodies leaves antibodies of anti-αvβ3 specificity behind (**left**). The eluate is reactive with both integrins, indicating the presence of anti-β3/anti-αIIbβ3 (**right**). One representative gel from independent experiments (n=9) is shown. OD indicates optical density.

In this study, we demonstrate that maternal sera from +ICH cases, but not –ICH cases, contain significant amounts of anti–HPA-1a antibodies, which exclusively react with the αvβ3 complex. Binding of this antibody subtype to ECs hinders endothelial adhesion to vitronectin, leads to cell anoikis, and interferes with angiogenesis in an ROS-dependent manner. Our results indicate that the presence of the anti–αvβ3 subtype in maternal serum is the critical cause for the development of fetal ICH in FNAIT.

It is known that the polymorphic residue Leu33Pro residing on the PSI domain of the β3-integrin subunit controls the formation of HPA-1a epitopes.[9] However, little is known about the contribution of αIIb and αv subunits to the formation of these epitopes. Recent studies demonstrated that some anti–HPA-1a

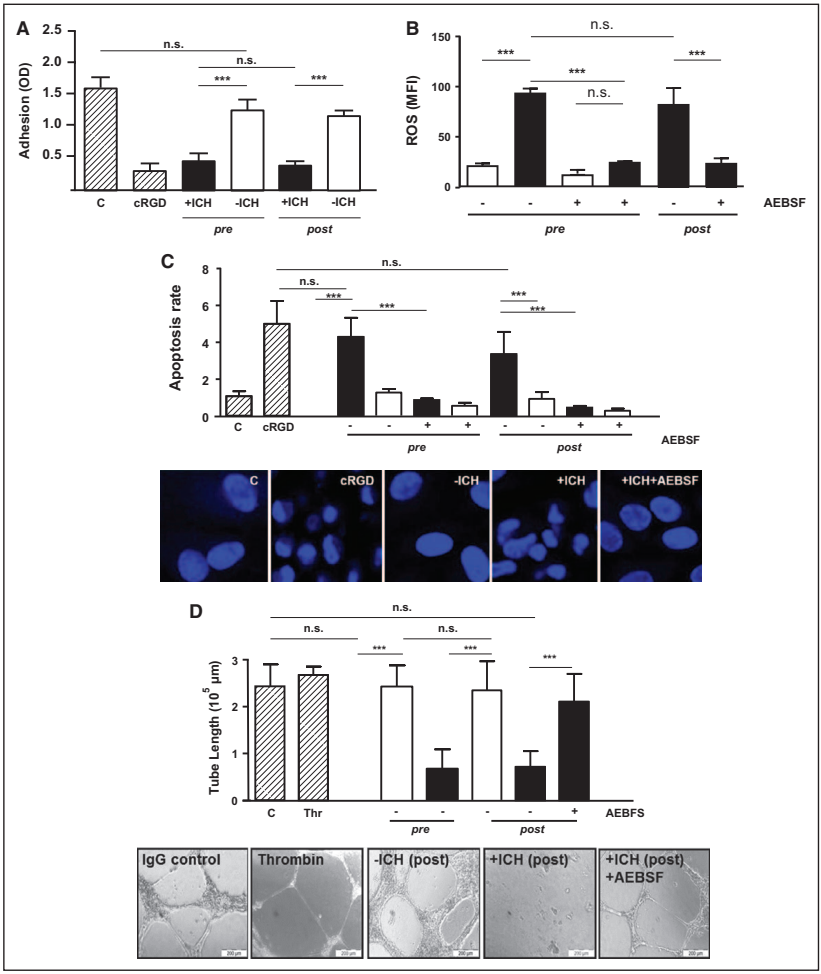

Figure 3. The αvβ3-specific subtype of anti–human platelet antigen 1a (HPA-1a) interferes with endothelial function. IgG was purified from maternal sera from +intracranial hemorrhage (ICH) cases (n=9; black columns) and from –ICH cases (n=9; white columns) either before (pre) or after (post) absorption with αIIbβ3 beads and then further studied. **A**, Cell adhesion. Endothelial cells were incubated with purified IgG (20 µg/mL) and added onto microtiter wells precoated with vitronectin. After washings, adherent cells were stained with crystal violet and measured using an ELISA reader. Statistical analysis was performed by 1-way ANOVA followed by Bonferroni post hoc test. Cyclic Arg-Gly-Asp peptide (cRGD) was used as a positive control. Note that only antibodies from +ICH cases hinder endothelial cell adhesion, with no difference between pre and postabsorption experiments. **B**, Reactive oxygen species (ROS) production. Endothelial cells were incubated with purified IgG (20 µg/mL) in the presence of DCFDA (2',7'-dichlorodihydrofluorescein diacetate). The production of fluorescent DCF (2',7'-dichlorofluorescein) correlates with cellular ROS production and was measured by flow cytometry. AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride) was used as an antioxidant in some experiments, as indicated. Statistical analysis was performed by 1-way ANOVA followed by Bonferroni post hoc test. Note that only antibodies from +ICH cases induce endothelial ROS, with no difference between pre and postabsorption experiments. **C**, Cell apoptosis. Purified IgG (20 µg/mL) was added to endothelial monolayers, and caspase 3/7 activity was measured by luminometry (**top**). Some experiments were performed in the presence of an ROS inhibitor (AEBSF). Statistical analysis was performed by 1-way ANOVA followed by Bonferroni post hoc test. cRGD was used as positive control. Note that only antibodies from +ICH cases induce apoptosis, with no difference between pre and postabsorption experiments. Nucleus staining of endothelial cells with DAPI after incubation with anti–HPA-1a IgG postabsorption and controls was performed (**bottom**). Representative pictures (60-fold magnification) from independent experiments (n=9) per cohort are shown. **D**, Tube formation. Purified IgG (40 µg/mL) was added to endothelial cells, and tube formation was investigated by microscopy. Data are given as mean of tube length in µm+SD (**top**). (*Continued*)

Figure 3 Continued. Some experiments were performed in the presence of an ROS inhibitor (AEBSF) as indicated. Statistical analysis was performed by 1-way ANOVA followed by Bonferroni post hoc test. Note that only antibodies from +ICH cases reduce tube length, with no difference between pre and postabsorption experiments. **Bottom**, Representative microphotographs of endothelial tube formation assays as outlined. C indicates control IgG from healthy donors; MFI, mean fluorescence intensity; n.s., not significant; OD, optical density; and Thr, thrombin.

antibodies bound discretely to the β3 chain, and some recognized complex (or compound) epitopes formed by αIIb and β3.[22–25] Similar to αIIbβ3, point mutation Leu33Pro together with αv subunit may create HPA-1a compound–dependent HPA-1a antigenic determinants. Our structural analysis of the PSI domain of αIIbβ3 and αvβ3 shows distinct conformational states that could in theory be differentially recognized by the immune system (Figure II in the online-only Data Supplement). However, other mechanisms may play a role.

We found in our cohorts a new anti–HPA-1a antibody subtype that reacts with compound epitopes formed by αv and β3 subunits. Accordingly, 3 different anti–HPA-1a subtypes may exist: anti-αIIbβ3 (reactive with platelets only), anti-β3 (reactive with platelets and ECs), and anti-αvβ3 (predominantly reactive with ECs). The third subtype is produced by FNAIT mothers with fetal/neonatal ICH.

It has become evident that integrin αvβ3–mediated adhesion to extracellular matrix is essential for EC growth and survival, whereas αvβ3 antagonism can induce endothelial apoptosis during angiogenesis.[26] In fact, disruption of αvβ3 ligation with monoclonal antibodies or peptide antagonist of αvβ3 caused detachment-induced endothelial anoikis[19] via activation of caspase-3 cascade[27] and impaired thereby angiogenesis.[28–30] In line with these observations, we show in this study that only the anti–αvβ3 subtype of anti–HPA-1a (that impaired αvβ3 ligation) could induce endothelial anoikis and affect angiogenesis.

Recently, Yougbaré et al[16] demonstrated that anti–β3 antibodies induce ICH in pups by impairing angiogenesis in mouse model of FNAIT. In this model, maternal isoantibodies were developed by transfusion of β3 knockout mice with wild-type platelets, and ICH frequency was increased with subsequent immunizations. In humans, however, severity of FNAIT usually does not increase with subsequent pregnancies,[17] and a high rate of ICH reoccurrence is found in previous siblings with ICH.[7,8] Unfortunately, analysis of different antibody subtypes was not investigated in this murine model. It is conceivable that, similar to humans, anti-αvβ3 impaired angiogenesis in these pups.

Vessel development in the brain depends on cross talk between ECs and perivascular astrocytes.[31] For this process, αv integrins play an important role through regulation of transforming growth factor-β activation and expression of transforming growth factor-β responsive genes that promote vessel differentiation and stabilization.[32–34] A critical period for ICH in FNAIT is before week 28 of gestation.[35] Of note, rapid development of premature medullary veins into shower-like numerous vessels associated with extensive angiogenesis has been observed between weeks 19 and 24 of gestation.[36] Therefore, it is feasible that antibodies against αvβ3 interfere with these critical processes to provoke ICH, especially in the brain within this period. To which extent thrombocytopenia further modifies bleeding probability and severity in the fetus requires further research.

In summary, our study provides evidence that human anti–HPA-1a of anti-αvβ3, but not of anti-β3, specificity can affect fetal vessel wall integrity, a mechanism that seems to be responsible for FNAIT-associated ICH. Our findings not only contribute to our understanding of FNAIT pathology in humans but also opens the way for new diagnostic testing and treatment strategies for immunized women in subsequent pregnancies. The absence of the anti–αvβ3 subtype could indicate a lower risk (or no risk) for ICH and might allow for modification (or cessation) of prophylactic FNAIT treatment. However, larger clinical studies are necessary to evaluate the clinical value of the proposed diagnostic test.

Acknowledgments

U.J. Sachs and S. Santoso designed the study. H. Wihadmadyatami, B. Bayat, S. Werth performed the experiments; H. Wihadmadyatami, T. Bakchoul, B. Bayat, N. Al-Fakhri, G. Bein, and P.J. Newman analyzed and interpreted the data. V. Kiefel and J. Zhu provided essential material. T. Bakchoul, B. Bayat, U.J. Sachs, and S. Santoso interpreted the data and wrote the article. We thank Heike Berghöfer (Giessen, Germany) for excellent technical assistance and K.T. Preissner (Giessen, Germany) for helpful discussions.

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Disclosures

None.

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Highlights

- Intracranial hemorrhage is the most serious complication of severe fetal/neonatal alloimmune thrombocytopenia, caused by maternal alloantibodies against human platelet antigen-1a.
- We show here that a specific anti–human platelet antigen-1a antibody subtype reactive with αvβ3 compound epitopes expressed on endothelial cells is present in intracranial hemorrhage cases.
- This antibody subtype impairs endothelial function, indicating that antiendothelial rather than antiplatelet activity is responsible for the development of intracranial hemorrhage in fetal/neonatal alloimmune thrombocytopenia.
- Our finding has the potential of changing diagnostic and therapeutic strategies in fetal/neonatal alloimmune thrombocytopenia.

Arteriosclerosis, Thrombosis, and Vascular Biology

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Antiendothelial αvβ3 Antibodies Are a Major Cause of Intracranial Bleeding in Fetal/Neonatal Alloimmune Thrombocytopenia

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Materials and Methods

Antibodies and peptides
Monoclonal antibody (moab) Gi5 against αIIbβ3 complex was characterized in our laboratory.[1] Moab 23C6 against αvβ3 complex[2] and moab P2W7 against αv^3 were purchased from Milipore (Temecula, CA, USA) and Calbiochem (La Jolla, CA, USA). Moab AP3 against the common β3 subunit[4] was produced by hybridoma AP3 cell line. RGD peptide was from Bachem, Bubendorf, Switzerland. Moab SZ21 specific against the HPA-1a alloform of β3 was from Beckman Coulter (Sinsheim, Germany). A soluble recombinant αIIbβ3 protein was produced in High Five cells.[5]

Serum samples
Maternal serum samples were selected from cases diagnosed with FNAIT in the Giessen laboratory based on the availability of adequate amounts of serum leftovers. For all selected samples, mothers were HPA-1a negative and had anti-HPA-1a antibodies, but no other HPA antibodies. Clinical data entries included neonatal brain ultrasound results, based on which the samples were assigned to the +ICH cohort (presence of ICH, n=18) or the –ICH cohort (absence of ICH, n=18). Demographic parameters (age, race) and clinical parameters as given in tables 1 and 2 including, neonatal platelet counts, were not statistically different between the +ICH and the –ICH cohort. From each cohort, 9 sera were chosen by list randomizer (http://www.random.org) for use in subsequent experiments. Serum samples from healthy blood donors were used as controls throughout the study. IgG fractions were purified using Melon Gel IgG Purification Kit as recommended by the manufacturer (Pierce Biotechnology, Rockford, IL, USA). IgG concentration was measured by Nano Drop (Thermo Scientific, Wilmington, DE, USA) and adjusted to a concentration of 20 µg/ml. Purified IgG was used immediately. Investigators in the laboratory were unaware of the clinical background of samples.

Endothelial cells
Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical vein as described[6] and maintained in endothelial basal medium-2 (EBM2; Lonza, Bazel, Switzerland). All experiments were performed with primary, secondary or tertiary post confluent monolayers of HPA-1aa or HPA-1bb genotyped HUVECs. HPA typing of HUVECs was performed by real-time PCR using VIC and FAM reporter probes (ABI Applied Bioscience, Washington, DC, USA) specific for HPA-1a and HPA-1b, respectively.

Stable cell lines expressing human αIIbβ3 and αvβ3 integrins
Chinese Hamster Ovary (CHO) cells expressing αIIbβ3 were generated as described.[7] CHO cells expressing the HPA-1a form of the αvβ3 integrin were a generous gift from Dr. Mark Ginsberg (University of California, La Jolla, CA, USA) and were cultured in DMEM medium (PAN) supplemented with 1% non-essential amino acids (Life Technologies), 1% penicillin/streptomycin (Life Technologies), and 10% FCS (PAN).

Characterization of HPA-1a alloantibodies by antigen capture assay
Aliquots of 30 µl platelets ($20x10^6$ cells) or HUVEC ($5x10^5$) were incubated with 20 µl serum and 10 µl moabs (concentration 20 µg/ml). Binding of human antibodies was analysed by MAIPA as described.[8] Cut-offs were calculated by the use of control sera. All experiments were run in triplicates, and results were given as arithmetic means of optical density (OD).

Production of αIIbβ3 coated beads
In brief, 2 ml amino-link resin beads (Thermo Scientific, Rockford, IL, USA) were coupled with 2 ml αIIbβ3 recombinant protein (0.35 mg/ml) in 200 µl coupling buffer overnight at 4°C.

Concentration of αIIbβ3 bound to the beads was measured by bicinchoninic acid assay (BCA; Thermo Scientific). To verify the specificity and absorption capacity of αIIbβ3 coated beads, 100 µl moabs specific for αIIbβ3 (clone Gi5), β3 (clone AP3), and αvβ3 (clone P2W7) (concentration 20 µg/ml) were absorbed with 100 µl αIIbβ3 beads (6.8 µg αIIbβ3/ml) overnight at 4°C. Absorbates were tested with αvβ3 and αIIbβ3 transfected CHO cells by flow cytometry (FACS Canto, Heidelberg, Germany) for the presence of remaining antibodies.

Separation of anti-HPA-1a subtypes from human sera
Aliquots of 100 µl αIIbβ3 beads were incubated with 100 µl serum overnight at 4°C. After centrifugation (1 min at 10,000g), supernatant was collected, and beads were washed twice with 500 µl isotonic saline. Antibodies bound to αIIbβ3 beads were eluted with 40 µl acid buffer (saline containing 1.5% BSA, and 1.0% acetic acid, pH 2.8) for 10 min at RT. Eluted antibodies were neutralized with 3 µl 2.5M Tris buffer, pH 7.2. IgG was purified as described IgG and was adjusted to a concentration of 20 µg/ml before use.

Analysis of different anti-HPA-1a subtypes by immunoprecipitation
CHO cells expressing αIIbβ3, αvβ3 or non-transfected CHO cells were surface labelled with 2 ml NHS-LC-Biotin (5 mmol/L; Pierce) as previously described.[24] Labelled cell lysates (100 µl) were incubated with 35 µl adsorbate or eluate (20 µg/ml) overnight at 4°C in the presence of 50 µl protein G beads (Gerbu Biotechnik, Heidelberg, Germany). After washings with immunoprecipitation buffer (50 mm Tris, 150 mm NaCl, 1% Triton X-100), bound proteins were eluted by adding SDS buffer for 5 min at 100 °C. Eluates were analyzed on 7.5% SDS-PAGE under reducing conditions. Separated proteins were transferred onto polyvinylidenfluorid (PVDF) membranes and developed with peroxidase-labeled streptavidin using chemiluminescence system (Immobilon Western Substrat, Millipore).

Apoptosis assay
Cell apoptosis was measured by the Caspase-Glo 3/7 assay (Promega, Madison, WI, USA). 100 µl vitronectin (2 µg/mL; Athens Research & Technology, Athens, GA, USA) were coated on 96 white well plates (Corning Incorporated, Corning, NY, USA) for 8 hours. Aliquots of 450 µl HUVEC (1-2x10^6 cells in EBM-2 medium) were incubated with cRGD (final concentration 40 µg/ml) or purified human IgG (final concentration 40 µg/ml) and seeded onto vitronectin coated wells for 16 hours at 37°C, 5% CO_2. 100 µl of Caspase-Glo 3/7 reagents were then added at RT and luminescence was measured using a fluorescence microtiter reader (FLX800, Biotek Instrument Winooski, VT, USA). In some experiments, 5 µl of 30 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF; Sigma) were added for 30 min at 37°C prior to addition of IgG.

Morphological assessment of apoptosis
Aliquots of 10^5 HUVEC were seeded onto µ-slide well (Ibidi, Martinsried, Germany) coated with vitronectin (see above) together with purified human IgG (final concentration 40 µg/ml) or cRGD (final concentration 40 µg/ml) for 16 - 18 hours at 37°C, 5% CO_2. After washings with 300 µl ice-cold PBS pH 7.4 (PAN, Aidenbach, Germany), cells were fixed with 300 µl 4% paraformaldehyde (PFA; Sigma) for 15 min and incubated with 1µg/ml blue fluorescing dye (Hoechst 33342; Thermo Scientific, Rockford, IL, USA) for 3 min at RT. Stained chromatin DNA was analysed using confocal microscopy with 60x magnification (Nikon Eclipse TE2000-E, Tokyo, Japan).

ROS detection assay
ROS produced in HUVEC was measured with 2',7'-dichlorofluorescein diacetate (DCFDA; Abcam, Cambridge, UK). In brief, 10^5 HUVEC were seeded in 6-wells plates for 6 hours and cultured overnight in EBM-2 medium containing 0.5% FCS. After removal of the medium, 1.5 mL medium containing DCFDA (final concentration, 10 mmol/L) was added. Subsequently, cells were incubated with mab against 23C6 or mouse IgG (5 µg/mL), cRGD (5 µg/mL), and anti-HPA-1a or control IgG (50 µg/mL) for 1 hour at 37°C. Cells were detached with accutase (Sigma), fixed by adding 250 µl CELLFIX (1:10 dilution; Becton Dickinson, Heidelberg, Germany), and measured by flow cytometry as described above. In some experiments prior to stimulation with antibodies, HUVEC were treated with AEBSF for 1 hour at 37°C.

Tube formation assay
Aliquots of 50 µl ice-cold matrix gel (Biovision, Milpitas, CA, USA) were plated onto microtiter wells (Greiner, Frickenhausen, Germany) for 30-60 min at 37°C. 100 µl HUVEC (1-5 x 10^5 cells in EBM2 medium supplemented with 2.5% FCS) were seeded carefully onto the gel for 45 min at 37°C. Thereafter, thrombin (1 U; Sigma, Steinheim, Germany) or IgG (40 µg/ml) were added. Cells were allowed to grow for 20 hours at 37°C. Data were analysed using a F-view monochrome fluorescence microscope (Olympus, Tokyo, Japan) with 10x magnification. For quantification, data were imported as TIFF files into ImageJ (http://imagej.nih.gov/ij/) using the stage micrometer as calibrator. In some experiments, 5 µl AEBSF was added for 30 min at 37 °C prior to addition of the antibodies.

Adhesion assay
Microtiter wells (Greiner Bio-one, Frickenhausen, Germany) were coated with 2 µg vitronectin (Athens Reasearch and Technology, Athens, GA, USA) or BSA (Serva, Heidelberg, Germany) in HBS buffer (119 mM NaCl, 4 mM KCl, 11 mM Glucose in 20 mM Hepes buffer) overnight at 4°C. After washings with HBS buffer, wells were blocked with 100 µl 3% BSA. Aliquots of washed HUVEC (1-4 x10^6) were added together with purified IgG or cRGD (final concentration 5 µg/ml) for 1 hour at 37°C. Wells were washed, adherent cells were stained with crystal violet (Sigma, Steinheim, Germany) and measured by a microtiter reader at 592nm (Sunrise™, Tecan, Maennedorf, Germany).

Structural analysis
The crystal structure of integrin αIIbβ3 extracellular domain (PDB code 3FCS) (ref: PMID: 19111664) was superimposed on the crystal structure of αvβ3 extracellular domain (PDB code 4G1M) (ref: PMID: 23106217) with PyMOL based on β3 PSI domain or β3 hybrid domain.

Statistical analysis
Statistical comparisons were made using an unpaired, 2-tailed Student's t test or 1-way ANOVA followed by Bonferroni's post-hoc test, as appropriate. A p-value <0.05 was assumed to represent statistical significance.

Study approval
The use of all human material was approved by the Ethics Committee of the Medical Faculty, Justus Liebig University, Giessen, Germany.

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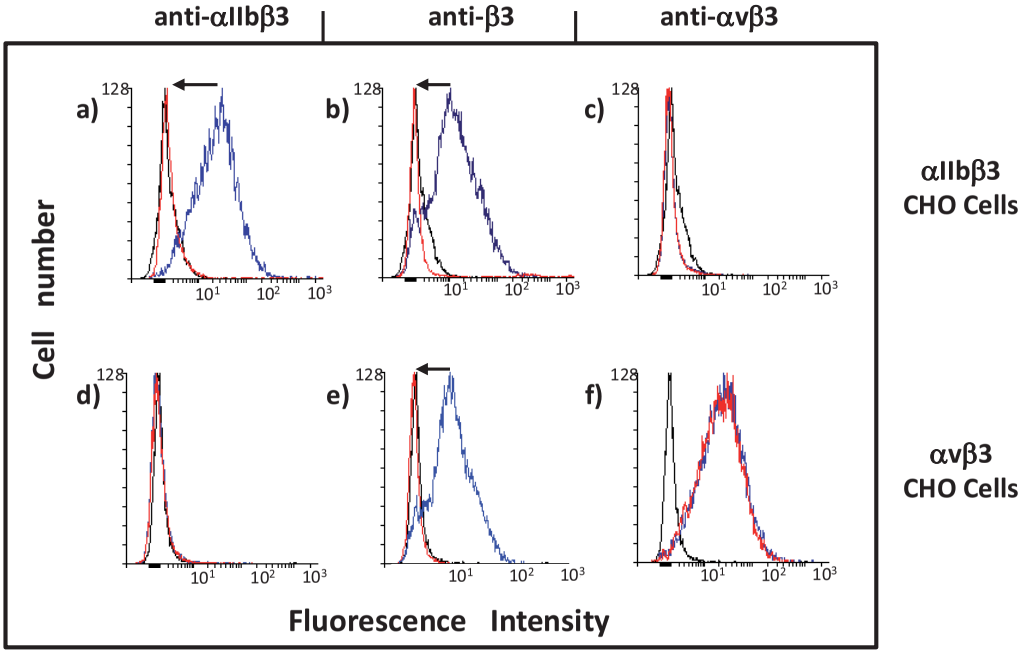
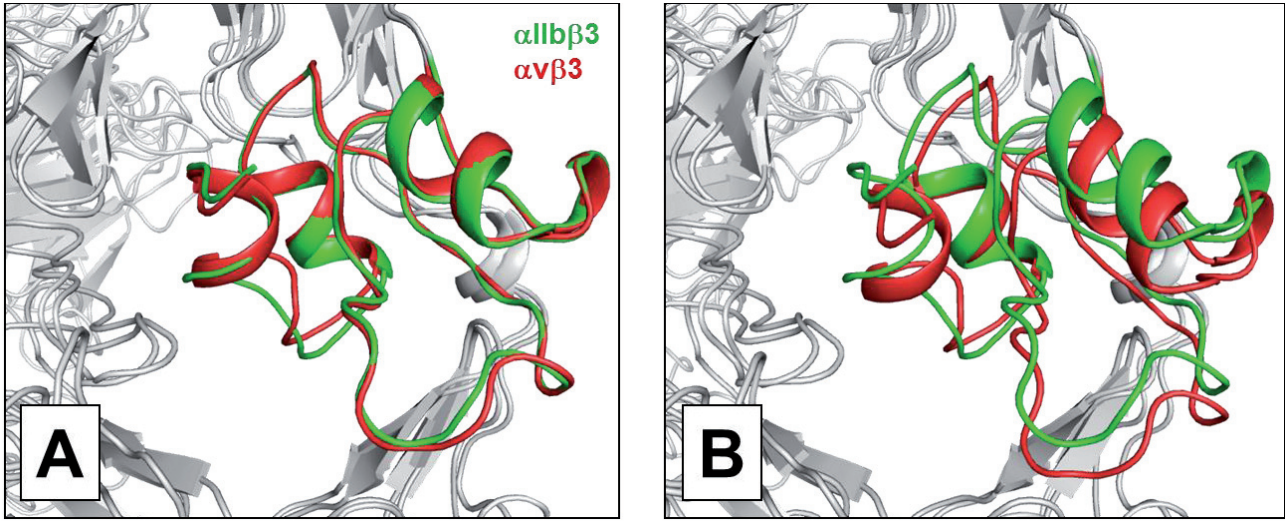
Supplemental fig. I

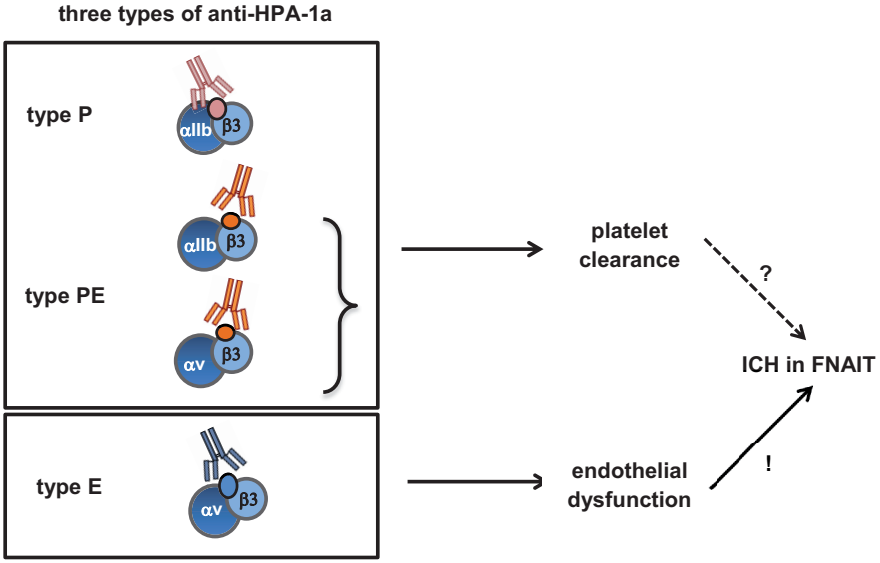


Identification of an anti-HPA-1a αvβ3-specific subtype in +ICH cases after absorption of other subtypes with αIIβ3 beads. Moabs against αIIbβ3 (a,d), β3 (b, e) and αvβ3 (c,f) were analyzed by flow cytometry before (blue) and after (red) adsorption with αIIbβ3 beads, using αIIbβ3 or αvβ3 transfected CHO cells as target. Isotype mouse IgG was used as control (black). Note that after absorption with αIIbβ3 beads, only anti-αvβ3 activity remains (f), whereas the reactivity against αIIbβ3 (a) and against β3 (b,e) disappears (indicated by arrows). Representative histograms from three independent experiments are shown.

Supplemental fig. II



Structural variation of the integrin β3 subunit PSI domain in the context of different a-subunit pairings. The PSI domains from αvβ3 and αIIbβ3 in isolation (panel A) are nearly superimposable, however when placed in the context of the entire β3 subunit that is paired with αv, versus the structure of the β3 subunit when paired with αIIb (panel B), the PSI domains adopt related, but distinct, conformational states that could easily be differentially recognized by the immune system (structures produced in PyMol freeware).


three types of anti-HPA-1a
type P
αIIb
β3
type PE
αIIb
β3
αv
β3
type E
αv
β3
platelet clearance
?
ICH in FNAIT
endothelial dysfunction
!

12. The Third Publication (Attachment 3)

Immunisation against αIIbβ3 and αvβ3 in a type 1 variant of Glanzmann's thrombasthenia caused by a missense mutation $Gly_{540}Asp$ on β3

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Summary

Treatment of bleeding in patients with Glanzmann's thrombasthenia (GT) can be hampered by iso-antibodies against the αIIbβ3 integrin, which cause rapid clearance of transfused donor platelets. Type 1 GT patients with a total absence of αIIbβ3 from the platelet surface are known to be susceptible to form such isoantibodies. In this study, we describe a type 1 GT patient with a missense mutation ($Gly_{540}Asn$) located in the EGF3 domain of the β3 integrin subunit. Cotransfection analysis in CHO cells demonstrates total absence of αIIbβ3 from the surface, based on inappropriate αIIb maturation. The patient's serum was reactive with αIIbβ3 and αvβ3 integrins in a capture assay, when platelets and endothelial cells were used. Two specificities could be isolated from the patient's serum, anti-αIIbβ3 and anti-αvβ3 isoantibodies. Both specificities did not interfere with platelet aggregation. In contrast, isoantibodies against αvβ3, but not against αIIbβ3, were able to disturb endothelial cell adhesion onto vitronectin, triggered endothelial cell apoptosis and interfered with endothelial tube formation. This intriguing finding may explain more recently observed features of fetal/neonatal iso-immune thrombocytopenia in children from type 1 GT mothers with intracranial haemorrhage, which could be related to anti-endothelial activity of the maternal antibodies. In conclusion, we give evidence that two isoantibody entities exist in type 1 GT patients, which are unequivocally different, both in an immunological and functional sense. Further research on the clinical consequences of immunisation against αvβ3 is required, predominantly in GT patients of childbearing age.

Keywords

Glanzmann's thrombasthenia, platelet function defect, isoimmunisation, αvβ3

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Introduction

Glanzmann's thrombasthenia (GT) is a rare autosomal recessive bleeding disorder caused by qualitative and/or quantitative deficiencies of αIIbβ3, which is the major fibrinogen receptor on platelets. These deficiencies lead to inadequate platelet aggregation in response to multiple physiological agonists. Based on laboratory criteria, GT patients may be sub-classified into type 1 GT (αIIbβ3 platelet surface expression, < 5%), type 2 GT (> 5–20%) and qualitative GT variants (1, 2). More than 150 naturally occurring mutations have been reported for the genes encoding αIIbβ3 (3, 4). A defect in either subunit (αIIb or β3) can alter the biosynthesis and/or structure of the αIIbβ3 heterodimer on the cell surface. GT patients have a lifelong moderate to severe haemorrhagic diathesis. Bleeding manifestations, which usually commence in early childhood, include easy bruising, petechiae, epistaxis, gingival bleeding, and menorrhagia. More severe spontaneous, unprovoked bleeding is uncommon in GT patients (5), but bleeding following trauma or invasive procedures, including minor procedures, may become life threatening (6). Most GT patients have a significant history of red blood cell and/or platelet transfusions, the latter of which can trigger the production of platelet isoantibodies against αIIbβ3, resulting in platelet transfusion refractoriness (7-10). Isoimmunisation of GT patients was also reported as a result of pregnancy (11, 12).

Notably, β3 also forms heterodimers with αv, both on platelets and endothelial cells (EC). Deficiency of αvβ3 has been reported for GT patients with β3 defects (13). The relevance of αvβ3 as an immuno-target in transfused GT patients has not been investigated so far. Here, we have identified a type 1 GT patient with a missense mutation on β3 ($Gly_{540}Asp$) who developed not only isoantibodies against αIIbβ3, but also against αvβ3. Anti-αvβ3 isoantibodies, but not αIIbβ3 isoantibodies, were capable of interfering with endothelial function and may be of particular relevance in isoimmunised GT mothers during pregnancy.

Material and methods

Case report

A 66-year-old male patient presented to the Haemostasis Centre after several episodes of critical peri-procedural bleedings requiring massive transfusions. Massive transfusion protocols were initiated during or after intervertebral disc surgery, a biopsy of the prostate and partial gastrectomy. These protocols included red blood cell, platelets, and plasma transfusions as well as the administration of prothrombin complex and fibrinogen concentrates. He presented himself to our institution 12 weeks after the last massive transfusion related to gastrectomy. All clinical material was obtained on that day. On admission, he had no signs or symptoms of bleeding. His Ivy bleeding time was 12 minutes (min) (reference less than 8 min). He had a normal coagulation screen and blood film, his platelet count was 150 G/l, platelet morphology was regular. In light-transmission aggregometry, his platelets were unable to respond to adenosine diphosphate (ADP 5–20 μM), collagen (4 μg/ml), arachidonic acid (250–500 mg/ml), and epinephrine (2–10 μM), but platelet agglutination in response to ristocetin (1.5 mg/ml) was normal. Informed consent from the patient was obtained as per guidelines of the institutional ethics committee.

Quantification of αIIbβ3 surface expression by flow cytometry

Platelet-rich plasma (PRP) was prepared from acid-citrate-dextrose (ACD) anticoagulated blood by centrifugation at 800 g for 20 min at room temperature (RT). The expression of glycoproteins α2β1, αIIbβ3, αvβ3 and GP Ib/IX on the platelet surface was quantified with platelet GP screen kit (Biocytek, Marseille, France) according to the manufacturer's instructions on a FACSCanto flow cytometer (Becton Dickinson, Heidelberg, Germany).

Platelet aggregation assay

PRP was prepared from ACD anticoagulated blood by centrifugation at 800 g for 20 min at RT. Platelet aggregometry was performed on a PAP-8 aggregometer (Moelab, Langenfeld, Germany) using collagen, adenosine diphosphate (ADP), arachidonic acid, epinephrine, and ristocetin (all from Moelab). In some experiments, antibodies were added prior to platelet aggregation.

Monoclonal antibodies and sera

Monoclonal antibodies (mabs) against αIIbβ3 complex (clone Gi5), against αIIb (clone Gi16) and against α2β1 (clone Gi19) were produced and characterised in our laboratory (14). Mabs SZ21 and SZ22 specific for αIIb and β3 subunit, respectively, were purchased from Beckman Coulter (Marseille, France). Mab against αvβ3 complex (clone 23C6) (15) and against αv subunit (clone P2W7) (16) were purchased from Millipore, Temecula, CA, USA. Mab against GAPDH was from Hyclon (South Logan, UT, USA). Anti-HPA-1a serum was obtained from a mother who gave birth to a child with severe neonatal alloimmune thrombocytopenia with intracranial haemorrhage. Serum from a healthy blood donor was used as control.

CHO cells expressing human β3 integrins

Chinese Hamster Ovary (CHO) cells expressing human αvβ3 integrin were a generous gift from Dr. Mark Ginsberg (University of California, La Jolla, CA, USA) and were cultured in DMEM medium (PAN-Biotech, Aidenbach, Germany) supplemented with 1% non-essential amino acids (Life Technologies; Carlsbad, CA, USA), 1% penicillin/streptomycin (Life Technologies) and 10% foetal calf serum (FCS; PAN-Biotech). CHO cells expressing αIIbβ3 were developed in our laboratory (17) and maintained as described above.

Nucleotide sequence analysis

Full-length sequencing of ITG2B and ITGB3 was carried out as described previously. Nucleotide sequences of PCR primers, sequencing, and reaction conditions are available upon request (18). Briefly, αIIb and β3 coding regions of genomic DNA were PCR amplified with primers corresponding to intronic sequences surrounding all exons of the ITG2B and ITGB3 genes. PCR was carried out using a Fast-Start High Fidelity PCR system (Roche Diagnostic Corporation, Indianapolis, IN, USA). Prior to sequence analysis, all PCR products were purified with a QIAquick PCR purification kit (Qiagen Sciences, Valencia, CA, USA). Automated sequence analysis was performed in both directions on a genetic analyzer (ABI 3100; Applied Biosystems, Foster City, CA, USA).

Construction of expression vectors

A full-length construct for the β3 mutant isoform was produced by site-directed mutagenesis of wild-type β3 in pcDNA3.1/Zeo mammalian vector using Quick Change Mutagenesis Kit (Strategene, Heidelberg, Germany) as previously described (17). For PCR amplification, site directed mutagenesis primers (5'-TGTGCTCAGGCCATGACCAGTGCAG CTGTGG-3' and 5'-CCACAGCTGCACTGGTCATGGCCTGAGCACA-3') were used (Eurofins MWG Operon, Ebersberg, Germany). After denaturation for 30 seconds (sec) at 95 °C, amplification consisted of 12 cycles (denaturation at 95 °C for 30 sec, annealing at 55 °C for 1 min, and extension at 65 °C for 12 min). PCR products were digested with Dpn endonuclease for 1 hour (h) at 37 °C, and transfected into DH5α high efficiency competent E. coli (Life Technologies). Plasmid DNA from positive clones was verified by nucleotide sequencing as described above.

Transfection of mutant and wild-type αIIbβ3 in mammalian cells

CHO cells (American Type Tissue Collection, Rockville, MD, USA) were grown in alpha-MEM medium (PAN-Biotech) supplemented with 10% foetal calf serum (FCS; PAN-Biotech) and were transfected with β3 (wild-type or mutant) together with αIIb using

Superfect Transfectant Reagent (Qiagen). After four weeks of cell culture in the presence of Zeocin and Geneticin (GIBCO BRL, Grand Island, NY, USA), cell lines were lysed and analysed for αIIbβ3 expression.

Immunoprecipitation and immunoblotting

In brief, transfected cells and platelets were surface labelled with 5 mmol/l NHS-LC-Biotin (Pierce, Rockford, IL, USA) and were precipitated as previously described (15). Labelled cell lysates were incubated with mab (20 μg/ml) or 50 μl serum overnight at 4°C in the presence of 50 μl protein G beads (Gerbu Biotechnik, Heidelberg, Germany). After washings with immunoprecipitation buffer (50 mm Tris, 150 mm NaCl, 1% Triton X-100), bound proteins were eluted by adding SDS buffer for 3 min at 100°C. Eluates were analysed on 7.5% SDS-PAGE under non-reducing conditions. Separated proteins were transferred onto polyvinylidenfluorid (PVDF) membranes and developed with peroxidase-labelled streptavidin using a chemiluminescence system (ECL, Amersham Biosciences, Glattbrugg, Switzerland).

Immunobloting was performed by using a total 200 μg/ml protein of unlabeled cell lysate. The lysate was separated on 7.5% SDS-PAGE under non-reducing conditions, transferred onto PVDF membranes, and stained overnight by incubation with a panel of mabs (final concentration, 15 μg/ml) and visualised by the use of peroxidase-labelled secondary antibody and an ECL system.

Characterisation of patient's antibodies by antigen capture assay

Platelets and HUVECs were used to characterise the presence of anti-αIIbβ3 and anti-αvβ3 isoantibodies in the patient's serum. Platelets were isolated from EDTA anticoagulated blood by differential centrifugation. Aliquots of 50 μl platelet suspension (2×10^6 cells) or 200 μl endothelial cells (5×10^5 cells) were incubated with 20 μl serum and 10 μl mabs (final concentration 20 μg/ml). Binding of anti-HPA-1a antibodies was analysed by MAIPA as described (19). Cut-off was calculated by the use of control sera. All experiments were run in duplicates, and results were given as arithmetic means of optical density (OD).

Separation of anti-αIIbβ3 and anti-αvβ3 isoantibodies using transfected cell lines

Aliquots of 1×10^6 CHO cells expressing αIIbβ3 or αvβ3 were incubated with 100 μl of patient's serum for 30 min at 37°C. After centrifugation for 1 min at 10,000 g, supernatant containing unbound antibodies (termed absorbate) was collected. Cells were then washed twice with 500 μl isotonic saline and resuspended in 80 μl isotonic saline. In order to obtain an eluate, cell-bound antibodies were eluted with 40 μl isotonic saline containing 1.5% BSA, and 1.0% acetic acid, pH 2.8 for 10 min at RT. Eluates were neutralised with 3 μl 2.5M Tris buffer, pH 7.2. From both absorbates and eluates, IgG was purified using Melon™ gel IgG spin purification kit (Thermo Fisher Scientific, Rockford, IL, USA). IgG concentration was measured by Nano Drop (Thermo Scientific, Wilmington, DE, USA) and adjusted to a concentration of 20 μg/ml. Purified IgG was used immediately. In some cases, sera were absorbed with αIIbβ3 coated beads. In brief, 100 μl αIIbβ3 beads (6.8 μg αIIbβ3/ml) were incubated with 100 μl serum overnight at 4°C. After centrifugation (1 min at 10,000g), supernatant (termed absorbate) was collected, and beads were washed twice with 500 μl isotonic saline. Antibodies bound to αIIbβ3 beads were eluted as described above (termed eluate).

Adhesion assay

Microtitre wells (Greiner Bio-one, Frickenhausen, Germany) were coated with 2 μg fibrinogen (Calbiochem, Darmstadt, Germany), vitronectin (Athens Research and Technology, Athens, GA, USA) or BSA (Serva, Heidelberg, Germany) in HBS buffer (119 mM NaCl, 4 mM KCl, 11 mM Glucose in 20 mM Hepes buffer) overnight at 4°C. After washings, wells were then blocked with 100 μl 3% BSA for 1 h at 4°C. Aliquots of washed HUVEC cells (1–4 $\times10^6$) were added together with mabs, purified IgG (final concentration 5 μg/ml) for 1 h at 37°C. Wells were washed once, and adherent cells were stained with crystal violet (Sigma, Steinheim, Germany) and measured in a microtitre reader at 592 nm (Sunrise™, Tecan, Männedorf, Germany).

Apoptosis assay

Cell apoptosis was measured by the Caspase-Glo 3/7 assay (Promega, Madison, WI, USA). One hundred μl vitronectin (2 μg/ml; Athens Research & Technology) were coated on 96 white well plates (Corning Incorporated, Corning, NY, USA) for 8 h. Aliquots of 450 μl HUVEC suspensions ($1–2\times10^6$ cells in EBM-2 medium) were incubated with mabs against αIIbβ3, αvβ3 (final concentration 20 μg/ml), cRGD (final concentration 20 μg/ml), or purified human IgG (final concentration 20 μg/ml) and seeded onto vitronectin coated wells for 16 h at 37°C, 5% CO_2. One hundred μl of Caspase-Glo 3/7 reagents were then added at RT and luminescence was measured using a microtitre reader (FLX800, Biotek Instrument, Winooski, VT, USA).

Morphological assessment of apoptosis

Aliquots of 10^5 HUVEC were seeded onto μ-slide well (Ibidi, Martinsried, Germany) precoated with vitronectin (see above) together with purified human IgG (final concentration 20 μg/ml) or cRGD (final concentration 20 μg/ml) for 16 h at 37°C, 5% CO_2. After washings with 300 μl ice-cold PBS pH 7.4 (PAN, Aidenbach, Germany), cells were fixed with 300 μl 4% paraformaldehyde (PFA; Sigma) for 15 min and incubated with 1 μg/ml blue fluorescing dye (Hoechst 33342; Thermo Scientific, Rockford, IL, USA) for 3 min at RT. Stained chromatin DNA was analysed using confocal microscopy with 60× magnification (Nikon Eclipse TE2000-E, Tokyo, Japan).

Tube formation assay

Aliquots of 50 µl ice-cold gel (Biovision, Milpitas, CA, USA) were plated onto microtitre wells (Greiner Bio-one, Frickenhausen, Germany) for 45 min at 37°C. One hundred µl HUVEC (1–5×10^5 cells in EBM2 medium supplemented with 2.5% FCS) were seeded carefully onto the gel for 45 min at 37°C. Thereafter, 100 µl EBM2 medium containing thrombin (1 U; Sigma, Steinheim, Germany) or mabs against αvβ3 and αIIβ3 (final concentration 40 µg/ml) or purified human IgG (final concentration 40 µg/ml) were added. Cells were allowed to grow for 20 h at 37°C. Data were analysed using a F-view monochrome fluorescence microscope (Olympus, Tokyo, Japan) with 10× magnification. For the quantification of tube length, data were imported as TIFF files into ImageJ (http://imagej.nih.gov/ij/) using the stage micrometer as calibrator.

Alignment analysis for the IEGF-3 domain of β3

Nucleotide sequences from men, monkey, rabbit, cow, swine, mouse, and *Drosophila melanogaster* were derived from NCBI Gene Bank (NG_008332.1, XM_002834317.3, AF116270.1, JX475452.1, AF170527.1, BC125518.1, and L13305.1) and aligned with MEGA 6 software[20].

Statistics

Statistical comparisons were made using an unpaired, two-tailed Student's t-test or one-way ANOVA followed by Bonferroni's post-hoc test, as appropriate. A p-value <0.05 was assumed to represent statistical significance.

Study approval

The use of all human material was approved by the Ethics Committee of the Medical Faculty, Justus Liebig University, Giessen, Germany.

Results

Expression analysis of β3 integrin on patient's platelets

►Figure 1A shows the flow cytometry analysis of patient's platelet compared with a normal donor. In comparison to normal platelets, very low αIIbβ3 (1,053 ± 544 copies/platelet, n=3) and αvβ3 (755 ± 180 copies/platelet, n=3) expression were measured on the cell surface expression of our patient's platelets. These levels, however, are below the limits of our test system as defined by isotype control (729 ± 650 copies/platelet). In contrast, normal α2β1 (4,600 ± 1,850, n=3) and GPIb/IX expression (30,418 ± 12,000, n=3; data not shown) were found. The normal values for αIIbβ3, αvβ3, α2β1 and GPIb/IX expression were 43,000 ± 12,000; 1,228 ± 400; 4,000 ± 2,800 and 38,000 ± 11,000 copies/platelet, respectively.

To confirm these findings immunoblotting analysis was performed (►Figure 1B). In comparison to normal platelets, only residual αIIb, but not β3 and αv subunits was detectable in cell lysates of patient's platelets. Based on these results and platelet aggregation studies (see case report), type 1 GT was diagnosed.

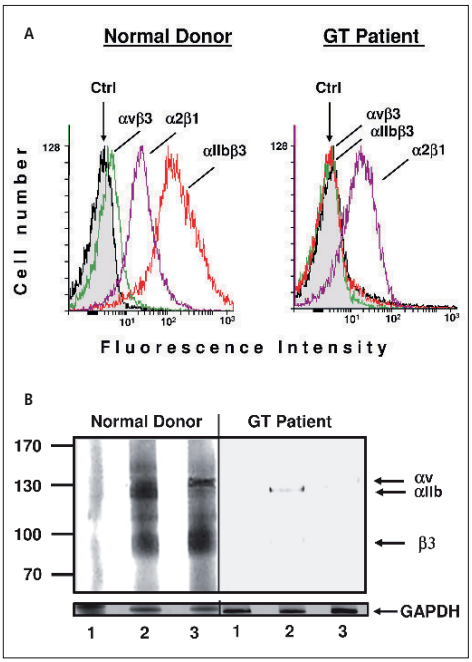


Figure 1: A) Quantification of glycoproteins on the platelet surface by flow cytometry. Normal donor platelets (left panel) and patient platelets (right panel) were incubated with monoclonal antibodies against αIIbβ3 (clone Gi5), αvβ3 (clone 23C6) and α2β1 (clone Gi19) as indicated. Mouse IgG was run as a control (Ctrl). Fluorescence-labelled donkey anti-mouse IgG was used as secondary antibody. **B) Immunobloting analysis of patient platelets.** Platelets from patient and normal donor were lysed, and aliquots of cell lysates (200 µg protein) were separated on 7.5% SDS-PAGE under non-reducing conditions. After blotting onto PVDF membrane, strips were subsequently incubated with mouse IgG (lanes 1), mabs against β3 (clone SZ21) and αIIb (clone SZ22) (lanes 2), or mabs against β3 (clone SZ21) and αv (clone P2W7) (lanes 3). Bound antibodies were then visualised using peroxidase-labelled secondary antibody and a chemiluminescence system.

Molecular analysis of αIIb (*ITGA2B*) and β3 (*ITGB3*) genes

To identify the molecular genetic defect in this patient, we isolated genomic DNA from the patient's leukocytes and amplified all exons of *ITGA2B* and *ITGB3* gene by PCR. Nucleotide sequencing analysis showed a homozygous G>A transition at position 1697 of

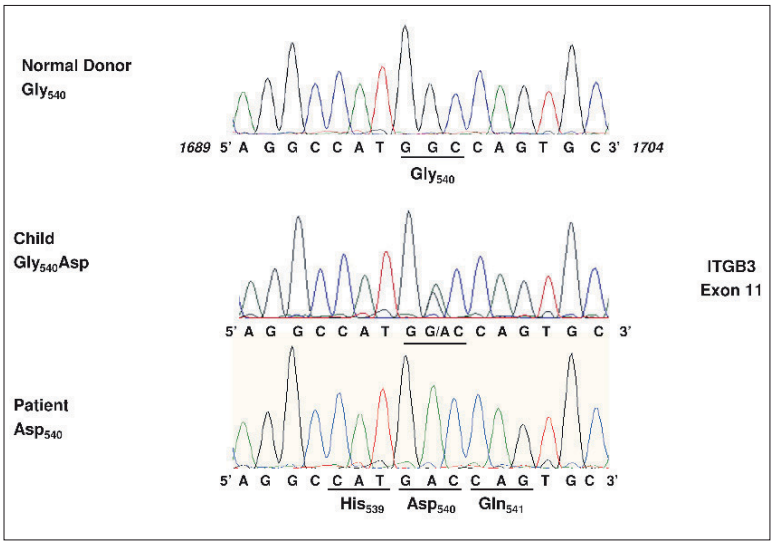


Figure 2: Nucleotide sequencing analysis of *ITGB3* gene. Results for nucleotides 1689 to 1704 are shown for a healthy individual, the GT patient, and one of his children. The nucleotide substitution G>A at position 1697 in exon 11 and the corresponding amino acid change from Gly540 (GGC) to Asp540 (GAC) are indicated.

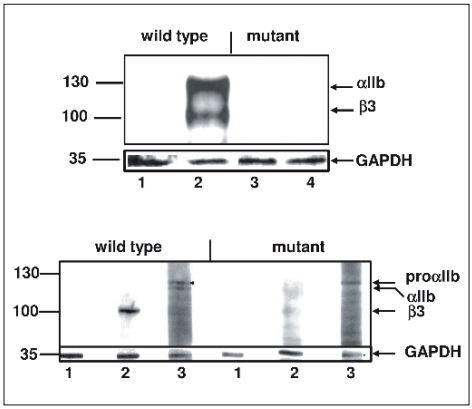


Figure 3: Immunochemical analysis of CHO cells transfected with mutant β3. Upper panel: CHO cells were transfected either with wild-type β3 or mutant β3 together with wild-type αIIb constructs. Cells were surface labelled with biotin and precipitated with mouse IgG (lanes 1 and 3) and mabs against αIIbβ3 (lanes 2 and 4). Immunprecipitates were separated on 7.5% SDS-PAGE under non-reducing conditions and transferred onto PVDF membrane. Bands were then visualized using peroxidase-labelled streptavidin and a chemiluminescence system. Bottom panel: Transfected CHO cells (as above) were lysed, and cell lysates were separated on 7.5% SDS-PAGE under non-reducing conditions. After blotting onto PVDF, strips were incubated with mouse IgG (lanes 1), mabs against β3 (lanes 2) and αIIb (lanes 3), and visualised as described in Figure 1B.

ITGB3, leading to a missense substitution Gly540Asp (GGC>GAC) in the I-EGF3 domain of β3 (►Figure 2). Sequencing the entire αIIb coding region showed no deviation from the wild-type form (not shown).

Effect of the Gly540Asp mutation on αIIbβ3 expression

To prove whether the Gly540Asp mutation located on β3 is directly responsible for the lack of αIIbβ3 surface expression, full-length cDNA constructs encoding for the mutated β3 form were generated. A mutated β3 construct was transfected together with wild-type αIIb into CHO cells, and αIIbβ3 expression on the surface of transfected cells was analysed by immunoprecipitation. As shown in ►Figure 3 (upper panel), CHO cells transfected with wild-type αIIb together with mutant β3 (Asp540) completely failed to express αIIbβ3 on the cell surface. Neither αIIb nor β3 specific bands could not be detected, even after prolonged exposure time (data not shown). Immunoblot analysis revealed that the mutant cells produced only pro-αIIb, which was not processed into mature αIIb, the obligatory step for αIIbβ3 surface expression. Anti-β3 detected multiple bands (lane 2), most likely breakdown products from the β3 integrin (►Figure 3, bottom panel).

Development of platelet αIIbβ3 and αvβ3 isoantibodies

To analyse the possible development of isoantibodies due to platelet transfusions, patient serum was analysed by antigen capture

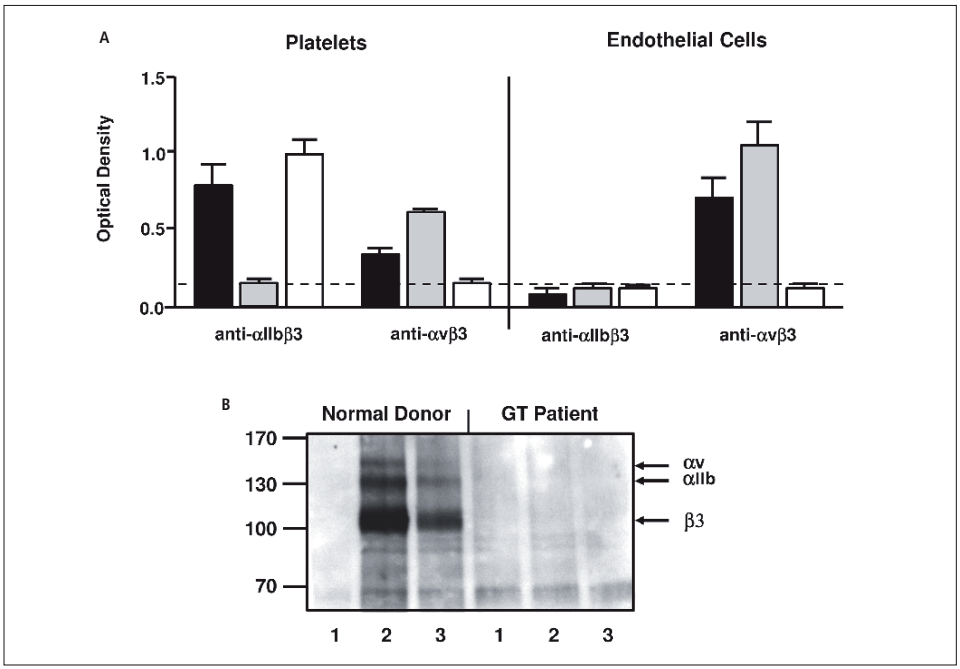


Figure 4: A) Analysis of isoantibodies by an antigen capture assay with platelets and endothelial cells. Platelets and endothelial cells (HUVEC) were incubated with patient serum (black columns) together with anti-αIIbβ3 (clone Gi5) or with anti-αvβ3 complex (clone 23C6). After lysis, the tri-molecular complex was captured by rabbit anti-mouse IgG. Human antibodies bound to immobilised αIIbβ3 or αvβ3 were detected with enzyme-labelled goat anti-human IgG and analysed by spectrophotometry (OD at 490/620 nm). Data are given as mean ± SD from n=3 independent experiments. In addition, patient serum was absorbed with αIIbβ3-coated beads and the absorbate (i.e. unbound antibodies remaining in the supernatant; grey columns) and the eluate prepared from the beads (i.e. antibodies initially bound to αIIbβ3-coated beads and then removed; white columns) were analysed (see Materials and methods for details). The dashed line represents the cut-off derived from the analysis of control sera (mean ± SD; n =3). **B) Immunoprecipitation analysis of patient platelets lysate.** Platelets from patient and normal donor were surface-labelled with biotin and lysed. Labelled platelet lysates were precipitated with control serum (lanes 1), anti-HPA-1a (lanes 2), and patient serum (lanes 3). Immunoprecipates were run on 7.5 % SDS-PAGE under non-reducing conditions. Separated proteins were transferred onto PVDF and visualised by the use of streptavidin and a chemiluminescence system.

assay using mabs αIIbβ3 and αvβ3 and platelet or endothelial cells as targets. As shown in ▶Figure 4A, patient serum reacted with platelet αIIbβ3 as well as platelet αvβ3 (black columns). When tested with endothelial cells, strong anti-αvβ3 reactivity was observed; whereas no anti-αIIbβ3 reactivity was detected (this integrin is not expressed on endothelial cells). To confirm the presence of anti-αvβ3 isoantibodies, patient serum was incubated with αIIbβ3-coated beads. After absorption with these beads, antibodies remaining in serum (absorbates; grey bars) still showed positive reaction against αvβ3 expressed on platelets and endothelial cells, but did no longer react with platelet αIIbβ3. Vice versa, analysis of antibodies eluted from the beads (eluates; white bars) showed strongly positive reactions with αIIbβ3 on platelets.

These findings indicate that this type 1 GT patient, in response to platelet transfusions, developed not only isoantibodies against αIIbβ3, but also against αvβ3. These results could be confirmed by immunoprecipitation analysis (▶Figure 4B). Similar to control serum (anti-HPA-1a from a FNAIT case with intracranial haemorrhage), patient's serum precipitated not only αIIbβ3, but also αvβ3 from normal platelets. As expected, both β3-integrin complexes could not be precipitated from patient platelets.

Functional properties of platelet αIIbβ3 and αvβ3 isoantibodies

To study the function of anti-αIIbβ3 and anti-αvβ3 isoantibodies, the two different antibody specificities were separated by

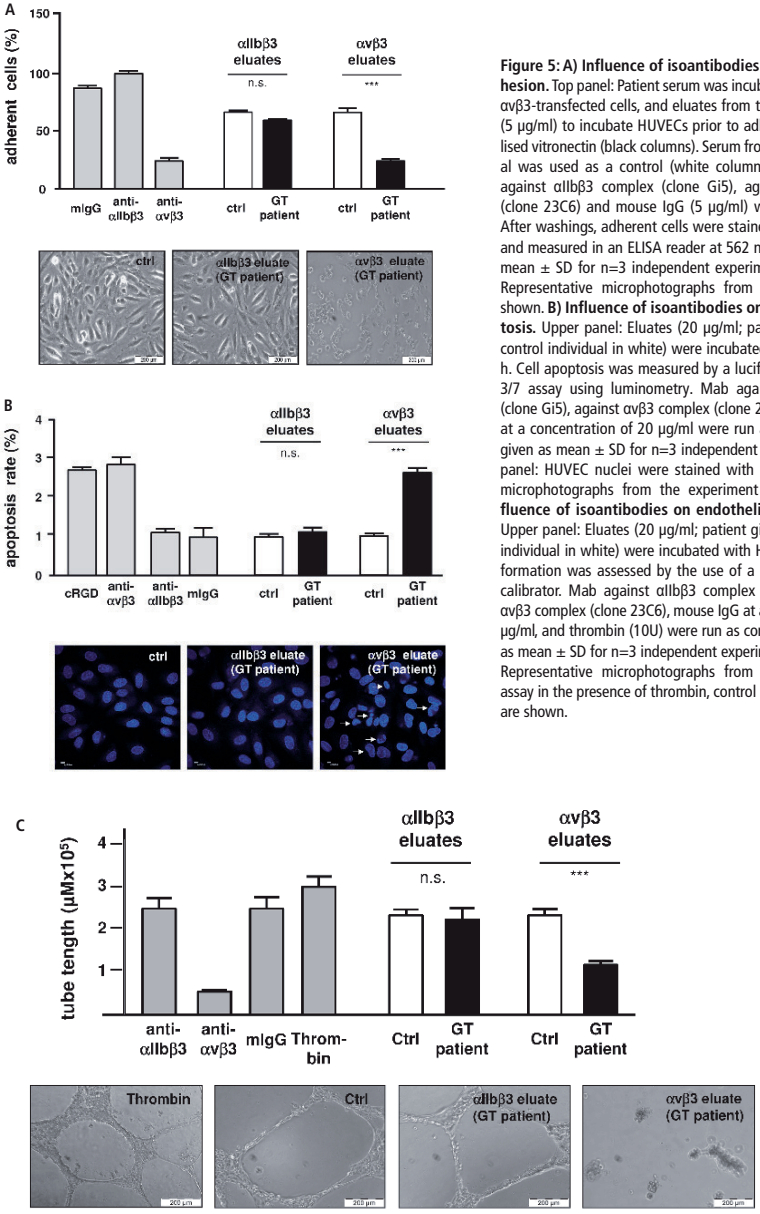
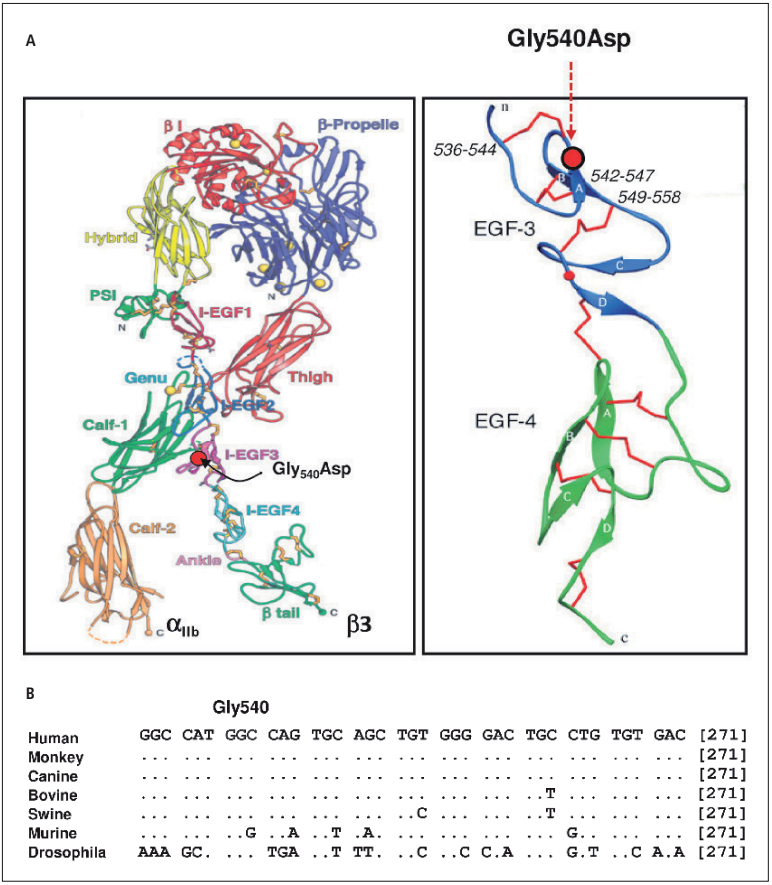


Figure 5: A) Influence of isoantibodies on endothelial adhesion. Top panel: Patient serum was incubated with αIIbβ3– or αvβ3-transfected cells, and eluates from these cells were used (5 µg/ml) to incubate HUVECs prior to adhesion onto immobilised vitronectin (black columns). Serum from a healthy individual was used as a control (white columns). In addition, mab against αIIbβ3 complex (clone Gi5), against αvβ3 complex (clone 23C6) and mouse IgG (5 µg/ml) were run as controls. After washings, adherent cells were stained with crystal violet and measured in an ELISA reader at 562 nm. Data are given as mean ± SD for n=3 independent experiments. Bottom panel: Representative microphotographs from the experiment are shown. **B) Influence of isoantibodies on endothelial apoptosis.** Upper panel: Eluates (20 µg/ml; patient given in black, control individual in white) were incubated with HUVEC for 18 h. Cell apoptosis was measured by a luciferase based caspase 3/7 assay using luminometry. Mab against αIIbβ3 complex (clone Gi5), against αvβ3 complex (clone 23C6) and mouse IgG at a concentration of 20 µg/ml were run as controls. Data are given as mean ± SD for n=3 independent experiments. Bottom panel: HUVEC nuclei were stained with DAPI; representative microphotographs from the experiment are shown. **C) Influence of isoantibodies on endothelial tube formation.** Upper panel: Eluates (20 µg/ml; patient given in black, control individual in white) were incubated with HUVEC for 18 h. Tube formation was assessed by the use of a stage micrometer as calibrator. Mab against αIIbβ3 complex (clone Gi5), against αvβ3 complex (clone 23C6), mouse IgG at a concentration of 20 µg/ml, and thrombin (10U) were run as controls. Data are given as mean ± SD for n=3 independent experiments. Bottom panel: Representative microphotographs from the tube formation assay in the presence of thrombin, control IgG, αIIbβ3 and αvβ3 are shown.



Human	GGC	CAT	GGC	CAG	TGC	AGC	TGT	GGG	GAC	TGC	CTG	TGT	GAC	[271]
Monkey	...	...	...	...	...	...	...	...	...	...	...	...	...	[271]
Canine	...	...	...	...	...	...	...	...	...	...	...	...	...	[271]
Bovine	...	...	...	...	...	...	...	...	...	..T	...	...	...	[271]
Swine	...	...	...	...	...	...	..C	...	...	..T	...	...	...	[271]
Murine	...	...	..G	..A	..T	.A.	...	...	...	...	G..	...	...	[271]
Drosophila	AAA	GC.	...	TGA	..T	TT.	..C	..C	C.A	...	G.T	..C	A.A	[271]

Figure 6: A) Localisation of the Gly540Asp mutation within αIIbβ3 integrin. The localisation of the Gly540Asp mutation in a model of the straightened extracellular domain of αIIbβ3 is shown (left), as it is in further detail within the EGF3 domain (right). Figures are adapted from Zhu et al., 2008 (32) (with permission). **B) Extracted nucleotide alignment for the IEGF-3 domain of β3 from different species.** Alignment analysis was performed by using MEGA 6 software. Note that the codon for Gly540 is highly conserved.

absorbing the patient's serum with αIIbβ3 and αvβ3 transfected cells. Bound antibodies, either anti-αIIbβ3 or anti-αvβ3 isoantibodies, were then eluted from the cells and were tested in different functional tests including platelet aggregation/adhesion, endothelial cell adhesion, apoptosis, and tube formation. Both specificities did not interfere with platelet aggregation and platelet adhesion onto fibrinogen (data not shown). Interestingly, anti-αvβ3 isoantibodies altered endothelial cell function significantly; they inhibited the adhesion of endothelial cells to vitronectin (►Figure 5A), induced endothelial apoptosis (►Figure 5B) and interfered with angiogenesis (►Figure 5C). In contrast, anti-αIIbβ3 isoantibodies did not exhibit any of these properties. These results demonstrate that type 1 GT patients can develop anti-αvβ3 which is immunologically and functionally different from anti-αIIbβ3 isoantibodies.

Discussion

In the present study, we describe a patient with Glanzmann's thrombasthenia with a missense mutation Gly540Asp located on the β3 integrin subunit, leading to type 1 GT.

Analysis of the patient's platelets by flow cytometry did not show any expression of αIIbβ3 or αvβ3 on the cell surface. Only residual αIIb was found in a lysate from patient platelets. We could demonstrate by transfection analysis in mammalian cells that the Gly540Asp mutation is directly responsible for the absence of

αIIbβ3 expression on the cell surface. Apparently, Gly540 falls within a structurally important region of the β3 chain, since it is located in the EGF3 domain (▶Figure 6A), surrounded by three disulfide bonds, Cys536–544, Cys542–547 and Cys549–558 21. A nearby-mutation, Cys542Arg, has previously been reported to cause type 1 GT (22, 23). Furthermore, the amino acid Gly at position 540 is highly conserved across species (▶Figure 6B).

More recently, the same mutation was discovered in a type 1 GT patient (GT53) from Argentina (24). In contrast to our numbering (based on mature protein), the position of the amino acid exchange in this paper was stated as 566 (based on immature protein; initiating Met stated as position +1).

It is well-known that type 1 GT patients with virtually absent αIIbβ3 expression can develop isoantibodies against αIIbβ3, leading to platelet transfusion refractoriness (7). Mutations of β3 can, however, not only alter the expression of αIIbβ3, but also of αvβ3 (13, 23, 25). Whether defective αvβ3 contributes to bleeding severity in GT patients are currently unclear (1); and the question whether such patients can develop isoantibodies against αvβ3 was purely speculative so far. To answer this question, absorption studies using recombinant αIIbβ3 coated on agarose beads were performed (▶Figure 4A). When absorbate (i.e. unbound antibodies in the supernatant) was analysed, it did not show any reaction with platelet αIIbβ3, but was strongly positive with endothelial αvβ3. In contrast, the eluate (i.e. antibodies eluted from αIIbβ3-coated beads) exclusively reacted with platelet αIIbβ3, but not with platelet-derived or endothelial-derived αvβ3. These results could be further confirmed by immunoprecipitation with platelets from a normal donor, from which the patient's serum precipitated both, αIIbβ3 and αvβ3. Thus, we demonstrate for the first time that type 1 GT patients can develop not only anti-αIIbβ3, but also anti-αvβ3 iso-antibodies. Whereas anti-αIIbβ3 isoantibodies did not have functional impact on platelets or endothelial cells, anti-αvβ3 isoantibodies interfered significantly with endothelial functions. Accordingly, the two isoantibody entities in this patient are unequivocally different, both in an immunological and functional sense.

Theoretically, anti-αvβ3 iso-antibodies should not harm the GT patient himself. The question whether such antibodies are able to contribute to platelet transfusion refractoriness in addition to anti-αIIbβ3 remains speculative for the moment.

However, transfer of anti-αvβ3 iso-antibodies is a relevant clinical condition that needs to be considered during pregnancy. These antibodies can pass the placenta and induce fetal/neonatal iso-immune thrombocytopenia, a condition that shares clinical features with fetal/neonatal alloimmune thrombocytopenia (25). Siddiq et al. recently reported on maternal immunisation against platelet antigens in a large cohort of GT mothers (12); fetal and neonatal death occurred only in mothers with antibodies. Anti-αIIbβ3 isoantibodies were also identified in association with intracranial haemorrhage (ICH) in two severely thrombocytopenic newborns from GT type 1 mothers (11, 26). However, maternal isoantibodies against αIIbβ3 were also found in healthy newborns without any signs of bleeding (12, 27-29). Since we have demonstrated that anti-αvβ3 isoantibodies hold special properties of interfering with endothelial function, it is intriguing to speculate whether isoanti-αvβ3 plays a role in the development of severe iso-antibody mediated thrombocytopenia, especially in cases with ICH.

It should be noted that in some GT cases, in contrast to the case reported here, β3 missense mutations appear to have different impact on the expression and function of αIIbβ3 and αvβ3 (24, 30, 31). Theoretically, mutated β3 in these patients may trigger the development of autoantibodies against β3 integrins, the proof of which is currently lacking.

In conclusion, we have shown that in a type 1 GT patient with a β3 (Gly540Asp) mutation, platelet transfusions have led to the formation of anti-platelet iso-antibodies which include both, antibodies of anti-αIIbβ3 and anti-αvβ3 specificity, with distinctive functional properties. We assume that iso-immunisation against αvβ3 may occur regularly in type 1 GT patients. Further research on the clinical consequences of anti-αvβ3 iso-immunisation is required.

What is known about this topic?

- Patients with type 1 Glanzmann's disease are prone to develop isoantibodies against αIIbβ3, the major β3 heterodimer on the platelet surface, as a consequence of transfusion or pregnancy.
- These isoantibodies may result in platelet transfusion refractoriness or, during pregnancy, in fetal/neonatal iso-immune thrombocytopenia.
- Isoantibodies against αvβ3, the second β3 heterodimer on the platelet surface, are not characterised in GT patients so far.

What does this paper add?

- Here, for the first time, isoantibodies against αvβ3 are demonstrated in a patient with type 1 Glanzmann's disease.
- These isoantibodies are unequivocally different to anti-αIIbβ3 isoantibodies, both in an immunological and functional sense.
- Since these isoantibodies have significant impact on endothelial cell function, further research is warranted, above all in female GT patients of childbearing age.

Acknowledgements

We would like to thank the patient and all members of his family for their kind co-operation. This work was supported by the Deutsche Forschungsgemeinschaft (Excellence Cluster Cardiopulmonary System; to SS) and by the Else Kröner-Fresenius-Stiftung, Bad Homburg vor der Höhe, Germany (to UJS). We thank Dr. Srikanth Karnati (Institute of Anatomy and Cell Biology, Justus Liebig University, Giessen, Germany) for helpful discussions regarding the tube formation assay; and all members of the Haemostasis Center, especially Dr. Ronald Fischer and Mrs. Bärbel Böttner, and from the Platelet and Neutrophil Laboratory at the University Hospital Giessen and Marburg, Giessen, Germany, for their support in this project. We also thank Dr. Peter Newman (Blood Research Institute, Milwaukee, Wisconsin, WI, USA) for providing us with recombinant αIIbβ3.

Conflicts of interest
None declared.

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13. The Forth Publication (Attachment 4)

Human Monoclonal Antibody Against HPA-1a inhibits anti-HPA-1a Mediated Endothelial Disturbance

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Keywords: FNAIT,mab d-813, antenatal treatment

Conflict of interest: No conflict of interest to declare.

Extra Table

What is known on this topic:

- Intravenous IVIG has been used as the best choice for antenatal treatment of FNAIT patient with some restricted problem regarding capability to increase platelet count on fetal and neonatal
- In vivo experiment showed Fab fragment from mouse monoclonal anti-HPA-1a (SZ21) could replacing the binding site of mother antibody against HPA-1a and prevent platelet clearance in circulations
- Deglycosylated mouse monoclonal anti-HPA-1a (SZ21) pass through the placenta and give satisfied results to prevent platelets clearance by macrophages.

What this paper adds:

- Here, we used for the first time deglycosylated humanized chimeric monoclonal antibody against HPA-1a term as mab d-813
- Based on the in vitro experiment by using endothelial cells, mab d-813 could prevent endothelial disturbance, additional invivo analysis showed that d-813 may prevent platelet clearance mediated by maternal anti-HPA-1a
- These results give new possibility that d-813 can be used as a novel antenatal treatment of FNAIT

Abstract

In this study, we proved whether modified human monoclonal antibody against HPA-1a (mab 813), can prevent endothelial dysfunction induced by maternal anti-HPA-1a alloantibodies. For this purpose, we removed the N-linked carbohydrate component of mab 813 by digestion with N-glycanase. This modified mab 813 (termed d-813) has several advantages; can inhibit the binding of maternal anti-HPA-1a antibody, can pass placenta barrier, but cannot recognize by RES system. Thus, the use of such antibody for prenatal treatment of FNAIT is feasible (Bakchoul et al, 2013). Here, we could demonstrate that d-813 itself did not impair endothelial functions, but d-813 administration prevents endothelial dysfunction mediated by maternal anti-HPA-1a. In addition, our *in vivo* experiment using NOD/SCID mouse model showed that d-813 could also prevent platelets clearance induced by anti-HPA-1a antibodies. This work represents the first evidence for the use of modified mab as drug for prenatal treatment of FNAIT

Introduction

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is a serious condition which is occurs as a result of transplacentally maternal antibodies reactive with platelet antigens which have associated to fetal and neonatal thrombocytopenia. In healthy fetuses, the platelet count is approximately 150 × 10^9/L at 10 to 17 weeks of gestation and reaches 265 ± 59 × 10^9/L by 30 to 35 weeks of gestation, in contrast on the fetus and neonate which is suffering of FNAIT the platelet count less than 150 × 10^9/L and it is possible will significantly decreased until less than 20× 10^9/L. The incidence of FNAIT is estimated at 1 per 1500-2000 live births of neonates especially in Caucasian populations (Kjeldsen-kragh et al. 2016; Bussel 1997; Bussel et al. 2005). However, this prevalence rate does not include the incidence of miscarriage in FNAIT, since the rate of affected women is largely unknown. FNAIT carries significant risks including intrauterine growth restriction (IUGR), intrauterine fetal demise and the most serious risk of FNAIT is intracranial hemorrhage which may have incidence is 14 - 20% on symptomatic infant (Mueller-Eckhardt et al., 1989; Bussel et al., 1991, 2005; Kaplan et al., 1991, Bertrand et al., 2011 blood). ICH may lead to death or persistent neurological sequel on the baby (McQuilten et al. 2011; Symington & Paes 2010). Occurrence of FNAIT in primiparous women is more common up to 50% of cases, thus making the diagnosis and treatment of this disease are more difficult, furthermore ICH may also occur intrauterine, on the 20 weeks ages of pregnancy (Tiller et al. 2015; Sachs 2013), since ICH are occurring earlier its make antenatal treatment of FNAIT are very necessary to be applied.

To days, injection of intravenous IgG (IVIG) with and without corticosteroid and also intra uterine transfusion with compatible platelet (IUPT) are being done as an antenatal treatment of FNAIT. IVIG treatment is relatively effective. However, this therapy relies on a human derived product, thus its make an open chance to

blood transmitted disease. In addition until today remain unclear, how is the IVIG protective mechanism. In a few case treatment failures have been reported, clinical observation show that administration of IVIG already failed to increase the amount of foetal or neonatal platelets counts (Bussel & Primiani 2008; Esa & Hhh 2008; Kanhai et al. 2005; Giers et al. 2010). Administration of IUPT also have limitations, and IUPT may increasing the risk of iatrogenic fetal death (Birchall et al. 2003). Thus, there is still opening possibility to solve the weakness of FNAIT antenatal treatment.

Material and Methods

Monoclonal antibodies

Monoclonal antibodies (mabs) against αIIbβ3 complex (clone Gi5) was produced and characterized in our laboratory. Mab SZ22 and SZ21 was purchased from Beckman Coulters, Krefeld, Germany. Humanized mab SZ21 (against HPA-1a) were from Dongying Lida Pharmaceutical, Suzhou, China. Mab against αvβ3 complex (clone 23C6) was purchased from Millipore, Temecula, CA, USA. RGD peptide was from Bachem, Bubendorf, Switzerland. Deglycosylated mabs was produced by incubation of 150 µg IgG with 40 µl N-glycanase (500.000 Units/ml; New England Biolabs, Ipswich, MA, USA) for 2 hours at 37°C. Intravenous IgG (IVIG) was derived from Biotest Pharma GmbH (Dreieich, Germany).

Surface Plasmon Resonance

The surface plasmon resonance experiments were performed using a ProteOn XPR 36 Protein Interaction Array System (BioRad, Munich) equipped with a research-grade GLC ProteOn sensor chip. The ligand (mab 813) was immobilized covalently using amine-coupling chemistry according to the

suppliers' protocol. Briefly, four parallel channels of the chip surface were activated with a 1:1 mixture of 400 mM 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimid (EDAC) and 100 mM N-hydroxysulfoxuccinimide (s-NHS) at a flow rate of 30 µl/min for 300 s. The capturing antibody was immobilized at a concentration of 10 µg/ml in sodium acetate, pH 4.5 was immobilized on all channels at densities between 3200-3500 RU. Remaining active sides on the surfaces were blocked with 1 M ethanolamine. Rec. αIIbβ3 and αvβ3 at concentrations 2.4 µg/ml in running buffer (10 mM HEPES, 150 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$ and 0.005% TWEEN 20, pH 7.4), were injected simultaneously over channels 1 and 2 and 3 respectively, flow channel 4 was left blank to serve as a reference surface representing uncovered capturing antibody interactions.

Adhesion assay

Microtiter wells (Greiner Bio-one, Frickenhausen, Germany) were coated with vitronectin (Athens Research and Technology, Athens, GA, USA) or BSA (Serva, Heidelberg, Germany) in HBS buffer (119 mM NaCl, 4 mM KCl, 11 mM Glucose in 20 mM Hepes buffer) overnight at 4°C. After washings, wells were then blocked with 100 µl 3% BSA for one hour at 4°C. Aliquots of washed HUVEC cells (1-4 $x10^6$) were added together with mabs, purified IgG (final concentration 5 µg/ml) for 1 hour at 37°C. Wells were washed once, and adherent cells were stained with crystal violet (Sigma, Steinheim, Germany) and measured in a microtiter reader at 592nm (Sunrise™, Tecan, Männedorf, Germany).

Apoptosis assay

Cell apoptosis was measured by the Caspase-Glo 3/7 assay (Promega, Madison, WI, USA). 100 µl vitronectin (2 µg/mL; Athens Research & Technology, Athens, GA, USA) were coated on 96 white well plates (Corning Incorporated, Corning, NY, USA) for 8 hours. Aliquots of 450 µl HUVEC suspensions (1-2x10^6 cells in EBM-2 medium) were incubated with mabs (final concentration 20 µg/ml), and cRGD (final concentration 20 µg/ml), then seeded onto vitronectin coated wells for 16 hours at 37°C, 5% CO_2. 100 µl of Caspase-Glo 3/7 reagents were then added at room temperature and luminescence was measured using a microtiter reader (FLX800, Biotek Instrument Winooski, VT, USA).

Morphological assessment of apoptosis

Aliquots of 10^5 HUVEC were seeded onto µ-slide well (Ibidi, Martinsried, Germany) precoated with vitronectin (see above) together with purified human IgG (final concentration 20 µg/ml), mab (final concentration 20 µg/ml) and or cRGD (final concentration 20 µg/ml) for 16 hours at 37°C, 5% CO_2. After washings with 300 µl ice-cold PBS pH 7.4 (PAN, Aidenbach, Germany), cells were fixed with 300 µl 4% paraformaldehyde (PFA; Sigma) for 15 min and incubated with 1µg/ml blue fluorescing dye (Hoechst 33342; Thermo Scientific, Rockford, IL, USA) for 3 min at RT. Stained chromatin DNA was analysed using confocal microscopy with 60x magnification (Nikon Eclipse TE2000-E, Tokyo, Japan).

Tube formation assay

Aliquots of 50 µl ice-cold gel (Biovision, Milpitas, CA, USA) were plated onto microtiter wells (Greiner Bio-one, Frickenhausen, Germany) for 45 min at 37°C. 100 µl HUVEC (1-5x10^5 cells in EBM2 medium supplemented with 2.5% FCS)

were seeded carefully onto the gel for 45 min at 37°C. Thereafter, 100 µl EBM2 medium containing thrombin (1 U; Sigma, Steinheim, Germany) or mabs (final concentration 40 µg/ml) or mabs (final concentration 40 µg/ml) were added. Cells were allowed to grow for 20 hours at 37 °C. Data were analysed using a F-view monochrome fluorescence microscope (Olympus, Tokyo, Japan) with 10x magnification. For the quantification of tube length, data were imported as TIFF files into ImageJ (http://imagej.nih.gov/ij/) using the stage micrometer as calibrator.

Analysis of d-813 in NOD/Scid mouse

Human platelets (PLTs) from HPA-1ab donors were prepared. Briefly, blood was drawn into ACE and supplemented with PGE1 at 50 ng per mL. After 10 minutes at RT, blood was centrifuged at 200 × *g* for 10 minutes. Washed PLTs were prepared as described previously, resuspended in autologous human plasma at 2.0×10^9 per mL, supplemented with PGE1 to 50 ng per mL, allowed to rest for 30 minutes, and injected into the retroorbital plexus of age- and sex-matched NOD/SCID mice (Stock No. complexes, 001303; The Jackson Laboratory, Bar Harbor, ME). Thirty micrograms of mab d-813, 813 or control in 200 µL of sterile Dulbecco's phosphate-buffered saline was injected intraperitoneally (IP) immediately after introducing the human PLTs. After a blood sample was taken to establish baseline human PLT counts, 800 µg of maternally derived anti-HPA-1a or control IgG was injected IP. Blood samples of 20 to 50 µL were taken periodically via tail tip amputation into 1 mL of a 1:9 mixture of 3.8 percent sodium citrate/Tyrodes-HEPES buffer containing PGE1 at 50 ng per mL. The blood mixture was layered onto 2 mL of Fico/Lite PLTs (Atlanta Biologicals, Lawrenceville, GA) and centrifuged for 15 minutes at 350 × *g*, and the PLT layer (1 mL) added to 3.0 mL Tyrodes-HEPES buffer supplemented with 67 ng per mL

PGE1. PLTs were washed by centrifugation at 750 × *g* for 10 minutes, resuspended in 50 µL Tyrodes-HEPES buffer and the percentage of circulating human PLTs was determined by flow cytometry using FITC-labeled monoclonal antibody AP2.

Statistics

Statistical comparisons were made using an unpaired, 2-tailed Student's t test or 1-way ANOVA followed by Bonferroni's post-hoc test, as appropriate. A p-value <0.05 was assumed to represent statistical significance.

Results

N-glycan modification of chimeric monoclonal antibody 813 by using PNG-ase F

To modify the N-glycan the humanized mab813 were deglycosylated and performed by using PNGase F, additionally, SDS-PAGE followed by silver staining was prepared to analyzed the degradation of antibodies, and as we expected after glycosylation native 813 are migrate more slowly compare to d-813 (Figure 1).

Deglycosylated 813 (d-813) showed similar binding affinity to unmodified 813

To investigate whether removing N-glycan impaired the binding affinity of the antibody surface plasmon resonance (SPR) was performed. In this experiment, we compared untreated and modified 813. Sensor chips were coated with recombinant αIIbβ3 and $\alpha v\beta$3, followed by the injection of 813 and d-813 in the different concentrations. Antibody binding was analyzed during an

association phase and a dissociation phase. For 813 a KD was observed to αIIbβ3 and αvβ3 is 1.54 x 10^{-12} and 7.43 x 10^{-11}, which comparison to d-813 a KD was observed to αIIbβ3 and αvβ3 is 3.48 x 10^{-12} and 6.67 x 10^{-11} (Figure 2). Taking together, these data confirm that N-glycan removal did not impair the binding affinity of 813 and d-813 to the αIIbβ3 and αvβ3 integrin epitope.

d-813 prevent endothelial disturbance mediated by maternal anti-HPA-1a

The next experiment, we performed several functional assay to prove wheater d-813 may prevent endothelial disturbance mediated by maternal anti-HPA-1a. All of the experiment are repeated three times and performed by using monoclonal antibody and IgG fraction derived from αIIbβ3 coupling beads absorption method.

The first experiment we performed adhesion test to prove whether deglycosylated 813 can prevent the binding of endothelial cells onto vitronectin matrix in the presence of a-HPA1a. In comparison to well established inhibitors, cRGD and anti-Vitronectin receptor, both native and deglycosylated 813 did not inhibit the binding of endothelial cells to vitronectin, additionally by using several different concentration of d-813 (20, 40, 60, and 80 µg/ml), d-813 have capability to inhibit the effect of a-HPA-1a in the endothelial cells on the high concentration (Figure 3).

Furthermore, we performed caspase 3/7 assay to prove whether deglycosylated 813 is able to inhibit endothelial apoptosis. In this experiments, we used three FNAIT ICH positive sera and shown that the presence of d-813 can inhibit endothelial cells apoptosis induced by a-HPA1a (Figure 4A) additional experiment with the microscopy analysis showing also that d-813 decreasing the amount of endothelial cells nucleus fragmentation (Figure 4B).

Subsequently, we also performed tube formation assay to analyze whether the d-813 would also prevent anti angiogenic effect derived from maternal anti-HPA-1a. The results described that in contrast to the absence of d-813, the presence of d-813 is able to inhibit the anti-angiogenic effect of anti-HPA-1a (Figure 5A). Representative microphotographs are shown in Figure 5B (right panel).

Taken together, the d-813 bound into endothelial cells and may inhibits cellular detachment to vitronectin, prevent cell apoptosis (anoikis), and consequently keep the angiogenic formation from the endothelial cells.

d-813 could prevent platelets clearance mediated by maternal anti-HPA-1a

To prove whether deglycosylated 813 may induce human platelet clearance untreated and deglycosylated 813 together with anti-HPA -1a were injected to NOD/SCID mouse prior to injection with human platelets. Periodically Human platelet clearances were examined in different time periods (60, 120 and 300 minutes) by flow cytometry. Significant platelet clearance was observed with native 813 and anti HPA-1a. In contrast, d-813 did not induce platelet clearance similar to untreated control, and surprisingly giving d-813 together with anti-HPA-1a may prevent any platelet clearance (Figure 6).

Discussion

Currently to prevent the effect because of FNAIT especially to avoid severe thrombocytopenia and ICH in the neonates a lot of effort already conduct. Since 1988 until today, IVIG is widely used as compromising and effective antenatal therapy for FNAIT, IVIG seems to give more protection against ICH, even this treatment are not guaranteed without side effect, meanwhile also on the persistence severe thrombocytopenia conditions the failure of the treatment still

presence on high-risk ICH mother (Bussel et al. 1996; Bussel et al., 2011) that's why optimal treatment regimens remain to be determined (Pacheco et al. 2011; Radder et al. 2004; Rayment et al. 2011). In addition, the mechanisms of IVIG in the treatment of immune-mediated thrombocytopenia is somehow controversial and debatable until today only one evidence mention how is IVIG mechanism, Widiapraja, and coworkers proposed that IVIG may protect the cells by reducing the inflammation rate (Widiapradja et al. 2014; Widiapradja et al. 2012). Recently in the last few year, such a preventive strategy has been proving successful in the murine model. Administration of anti-integrin β3 sera before injection of integrin β3 positive platelets in an integrin β3-/- murine model of FNAIT prevented the formation of anti-platelet antibodies and reduced bleeding complications in newborn pups (Tiller et al. 2015). Currently, clinical trials regarding FNAIT prevention based on prophylaxis are underway to test the potential of immunity of anti-HPA-1a IgG to prevent HPA-1a immunization (Kjeldsen-kragh et al. 2016), however, for this project the numbers of women HPA-1a alloimmunized in pregnancy which is needed as plasma donor is relatively small. Also, immunizing HPA-1a-negative individuals with HPA-1a-positive platelets would result in rare immunizations because transfusion of HPA-1a positive platelets rarely stimulates an antibody response against HPA-1a. In addition, polyclonal anti-HPA-1a IgG would carry the risks of the human-derived product. Therefore, monoclonal antibodies (mAbs) specific for HPA-1a would be very interesting for replacing polyclonal antibody as a source for FNAIT treatment.

Here in our study, we try to generate deglycosylated chimeric human mabs specific for HPA-1a term as d813 as antenatal treatment of FNAIT. Our group results previously suggest that on in vivo experiments deglycosylated mouse monoclonal anti HPA-1a (clone NGMSZ21) may pass through the placenta and compete to replacing the binding site of maternal alloantibody, thus

may prevent fetus platelets clearance by macrophages (Bakchoul et al. 2013). However, this antibody are still fully derived from mouse which has more highly side effect consequences if fully applied to human, to eliminate the immunogenicity of the mabs using genetic engineering we generate chimeric humanized 813 by eliminating the Fc fragment from murine became human Fc fragment. Deglycosylated with endoglycosidase F was performed to manipulated sugar at the position Asn 297 with endoglycosidase F (Figure 8). Since It has become increasingly clear that the Fc-linked glycosylation pattern regulates effector functions of IgG (Jefferis, 2009), Thus deglycosylation of the N-glycan attached to Asn297 inhibits recognition by Fc receptors on macrophages (FcRI, FcRIIa, FcRIIIa) as well as its ability to activate complement factor C1q (Nimmerjahn & Ravetch 2008). In contrast IgG transported from the maternal circulation to the fetus by binding to the neonatal Fc receptor of FcRn that is expressed in the placental villous syncytiotrophoblast (Leach et al, 1996; Roopenian and Akiles, 2007) FcRn-mediated IgG transport does not require carbohydrate moieties on the Fc part of the antibody for binding or transplacental transport (Palmeira et al. 2012; Einarsdottir et al. 2013). Thus, removal of the N-glycan should not affect placental transport.

In line with our finding Eksteen et al. describe new mab derived from a single memory B cell isolated from a woman which have HPA-1a immunized during pregnancy named as 26.4. This antibody could inhibit binding of maternal polyclonal anti-HPA-1a and have high specificity and binding affinity to HPA-1a, but unfortunately the epitope of mAb 26.4 is not constrained to the PSI domain, but extends to several domains of integrin β3 in contrast d-813 have a finite or a limited number of epitopes on the b3 integrin. Additonally Ghevaert et al. (2008) also prove that mab B2G1 derived from the maternal B cells of an FNAIT cases by phage display can block the binding of maternal anti-HPA1a in the range 70%

to 95% in 18 cases of FNAIT. Base on this evidence we postulated that d-813 is HPA-1a specific antibodies have a sufficient affinity to block maternal antibodies to the HPA-1a epitope. Interestingly mAb 26.4 bound with higher affinity to integrin αvβ3 from trophoblasts (Eksteen et al. 2015), compared to another HPA-1a-specific, B2G1 (Ghevaert et al. 2008). Additionally by using surface plasmon resonance compared to the native 813, d-813 performed the same binding affinity both to the αIIbβ3 and $\alpha v\beta$3.

There is necessary to analysis the effect of d-813 both on the platelets and ECs, thus in vivo and in vitro experiment were performed. On the in vivo experiment by using NOD/SCID mouse model upon the presence of HPA-1a antibodies, we have shown that d-813 may prevent platelets clearance from circulation. Moreover in our in vitro models prior the presence of d-813, this antibody seems did not inhibit adhesion of the ECs into vitronectin matrix, did not induce any apoptosis and did not impaired tube formation in angiogenesis assay. When anti-HPA1a are adding to the ECs prior the presence of d-813, this antibody have the capability to prevent apoptosis and restore angiogenesis as well as on the presence of IVIG. These results convince the capability of d-813 to prevent endothelial dysfunction cause by HPA-1a antibodies which react to the $\alpha v\beta$3 on the ECs especially to the brain and trophoblast. Inconclusion our experiment results confirm the therapeutic potential of d-813 as an alternative to antenatal treatment of FNAIT even how is the mechanism of the antibodies to give protection to the platelets and ECs need to be work out.

Acknowledgments

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Figure legends

Figure 1. N-glycan modification of humanized 813 term as"d-813".

Monoclonal antibodies 813 was treated with endonuclease F for 45 minutes in 37^0C following by SDS-PAGE and visualized by silver staining. All of the glycan start at the position if Asp 297 were removed indicated by decreasing of d-813 molecular weight.

Figure 2. Binding analysis of d-813 to αIIbβ3 and αvβ3 integrins by Surface Plasmon Resonance(SPR)

Recombinant αIIbβ3 and αvβ3 proteins were immobilized on the sensor chip. Different concentrations of native and deglycosylated 813 were injected, and the binding was recorded in real-time. SPR analysis showed there is no differences binding affinity between native and deglycosylated 813 to the αIIbβ3 and αvβ3 integrins

Figure 3. Cell adhesion Analysis

Endothelial cells were incubated with monoclonal antibodies (mabs) 813, d813, mabs against αvβ3, and purified IgG from sera HPA-1a (20 µg/ml) and added to microtiter wells pre-coated with vitronectin. After washings, adherent cells were stained with crystal violet and measured in an ELISA reader. Statistical analysis was performed by 1-way ANOVA followed by Bonferroni's post-hoc test; n.s. = not significant. cRGD was used as a positive control anoikis. Representative microphotographs of cell adhesion assay as outlined are given in the lower panel.

Figure 4. Cell apoptosis.

Monoclonal antibodies (mabs) 813, d813, and purified IgG (20 µg/ml) was added to endothelial monolayers and caspase 3/7 activity was measured by luminometry (upper panel). Some experiments were performed in the presence of IVIG. Statistical analysis was performed by 1-way ANOVA followed by Bonferroni's post-hoc test; n.s. = not significant; C = control IgG from healthy donors. mabs against αvβ3 was used as positive control. Nucleus staining of endothelial cells with

DAPI after incubation with anti-HPA-1a IgG, d-813, IVIG and controls were performed with representative pictures (60 fold magnification) (lower panel).

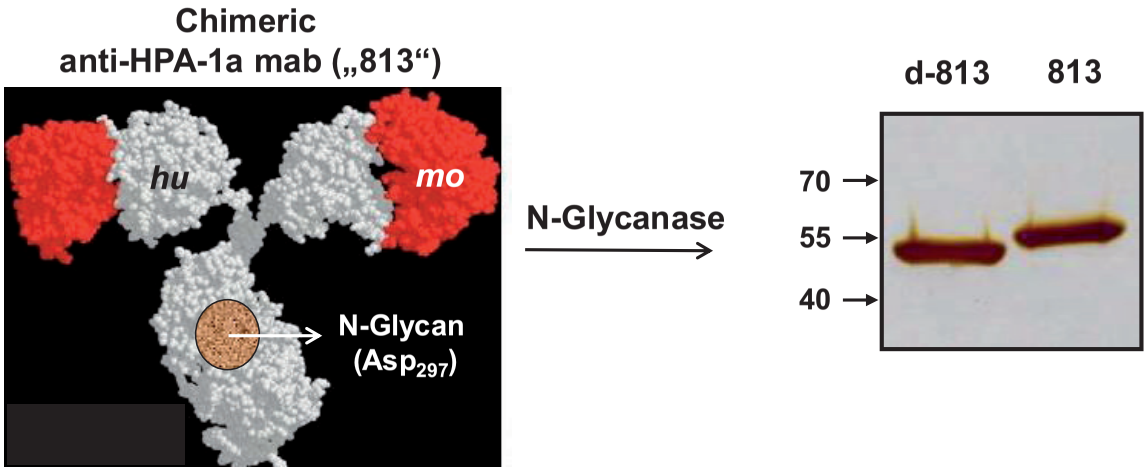
Figure 5. Tube formation analysis

Monoclonal antibodies (mabs) 813, d813, Purified IgG (40 µg/ml) was added to endothelial cells, and tube formation was investigated by microscopy. Data are given as mean of tube length in µm +SD (upper panel). Some experiments were performed in the presence of IVIG as indicated. Statistical analysis was performed by 1-way ANOVA followed by Bonferroni's post-hoc test; n.s. = not significant. C = control IgG; T = thrombin. Representative microphotographs of endothelial tube formation assays as outlined are given in the lower panel.

Figure 6. Mab d-813 prevents anti–HPA-1a antibody-mediated platelet destruction in a NOD/SCID mouse model of alloimmune thrombocytopenia.

Resting human platelets (HPA-1ab) were injected retroorbitally into NOD/SCID mice and the survival of platelets was analyzed. Results are shown as a median and range of experiments that were performed in duplicate with anti–HPA-1a antibodies from a FNAIT cases. When d-813 was injected before the maternal anti–HPA-1a antibodies, the elimination of platelets was largely inhibited (orange symbols), This finding also presents in the administrated of d-813 (black symbol) as well as the control antibody alone which did not induce any relevant platelet destruction (green symbols). However, the injection of maternal anti–HPA-1a and native 813 resulted in a rapid destruction of circulating human HPA-1a platelets (blue symbols).

Figure 1


Chimeric
anti-HPA-1a mab („813“)
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mo
N-Glycan
(Asp297)
N-Glycanase
d-813
813
70
55
40

Figure 2

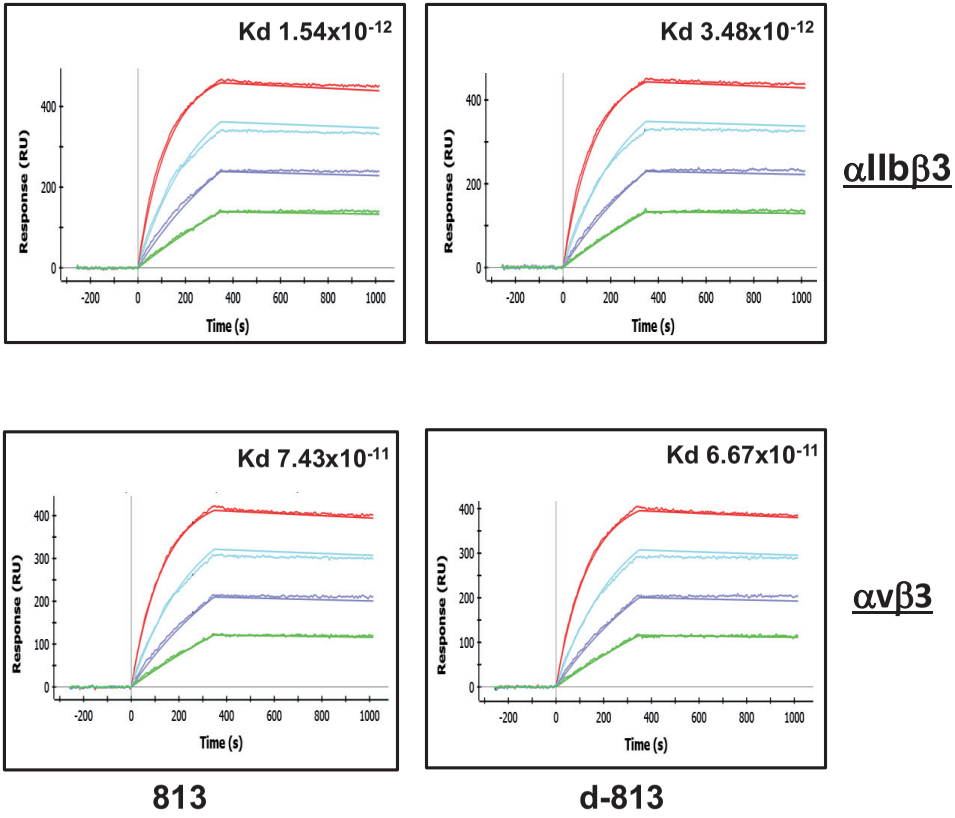

Kd 1.54x10-12
Kd 3.48x10-12
αIIbβ3
Kd 7.43x10-11
Kd 6.67x10-11
αvβ3
Response (RU)
Time (s)
813
d-813

Figure 3

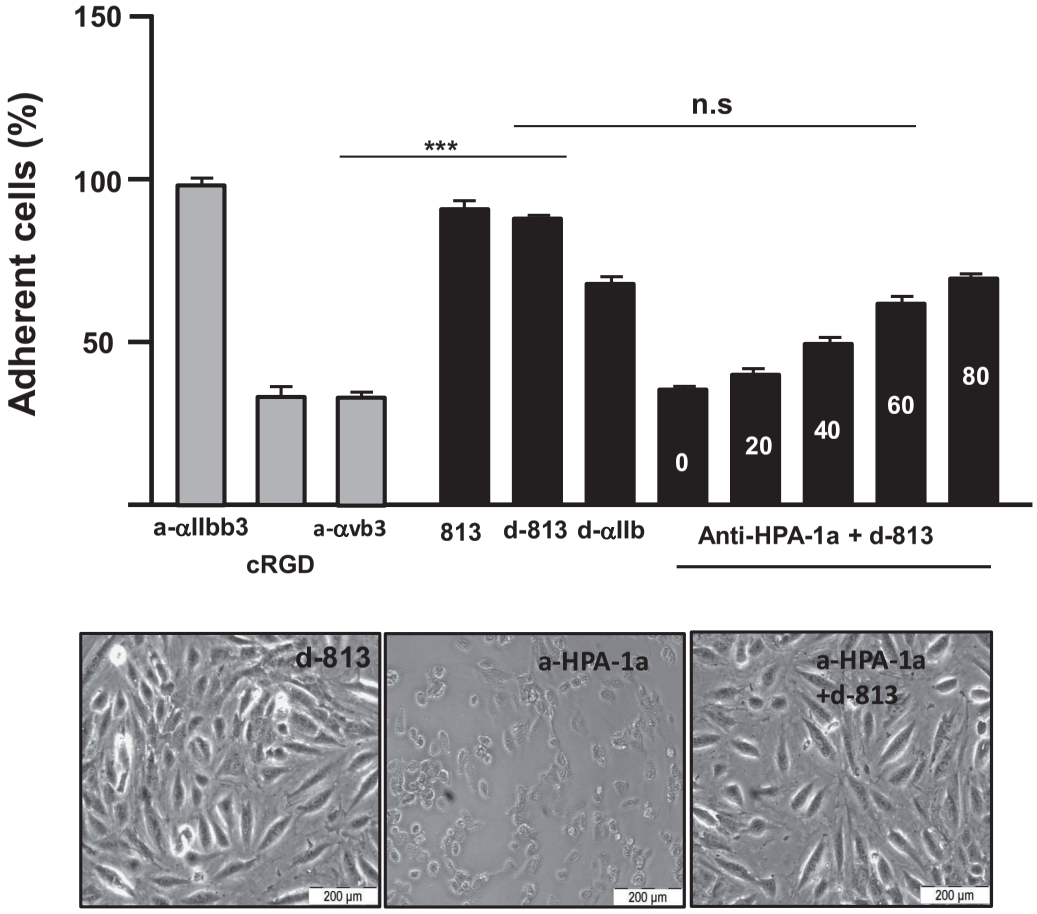
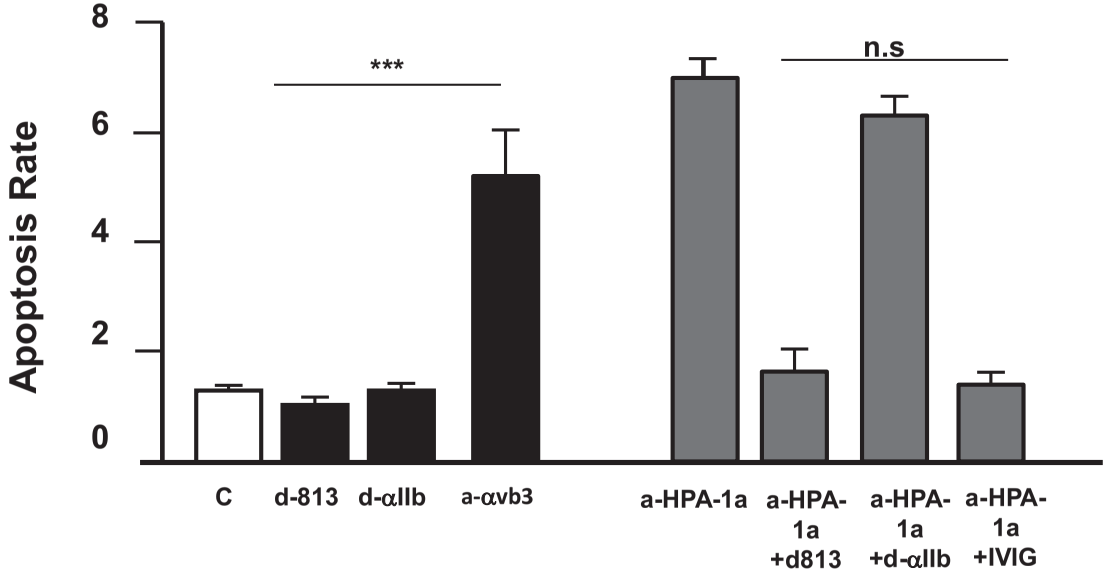

Adherent cells (%)
150
100
50
n.s
***
0
20
40
60
80
a-αIIbb3
cRGD
a-αvb3
813
d-813
d-αIIb
Anti-HPA-1a + d-813
d-813
a-HPA-1a
a-HPA-1a
+d-813
200 µm
200 µm
200 µm

Figure 4


Apoptosis Rate
8
6
4
2
0
***
n.s
C
d-813
d-αIIb
a-αvb3
a-HPA-1a
a-HPA-1a +d813
a-HPA-1a +d-αIIb
a-HPA-1a +IVIG

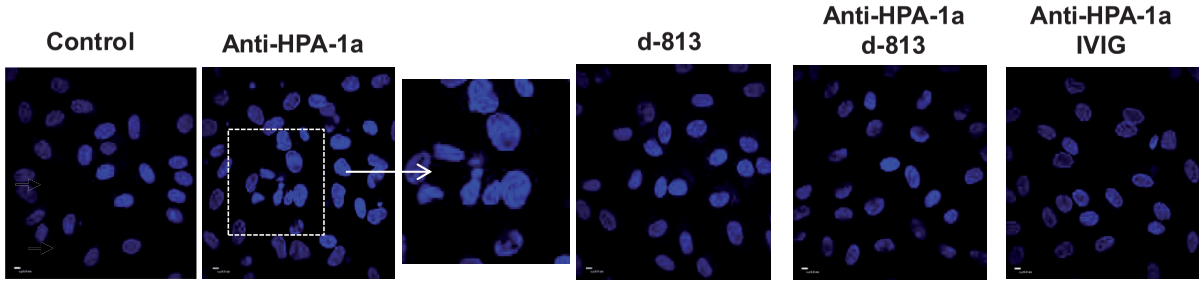
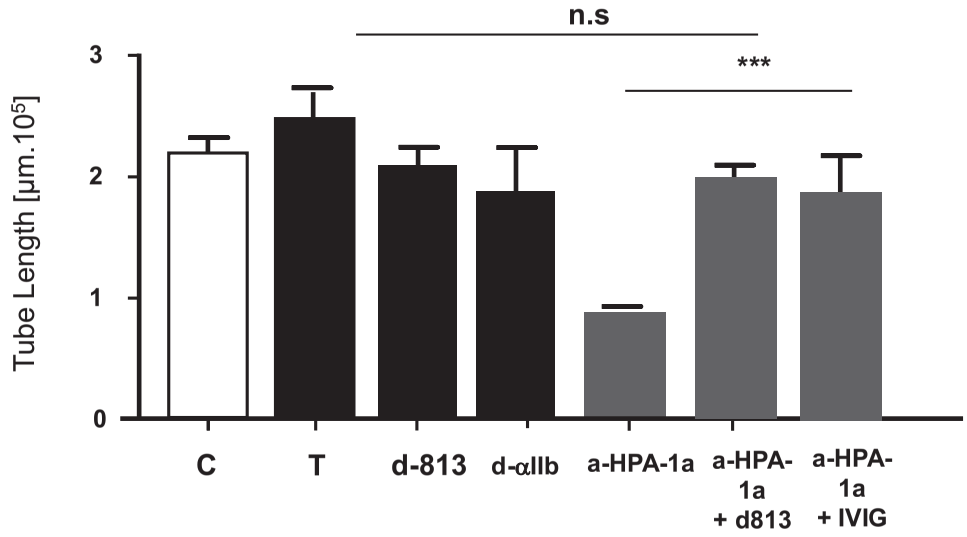

Control
Anti-HPA-1a
d-813
Anti-HPA-1a d-813
Anti-HPA-1a IVIG

Figure 5


n.s
***
Tube Length [µm.10^5]
3
2
1
0
C
T
d-813
d-αIIb
a-HPA-1a
a-HPA-1a + d813
a-HPA-1a + IVIG

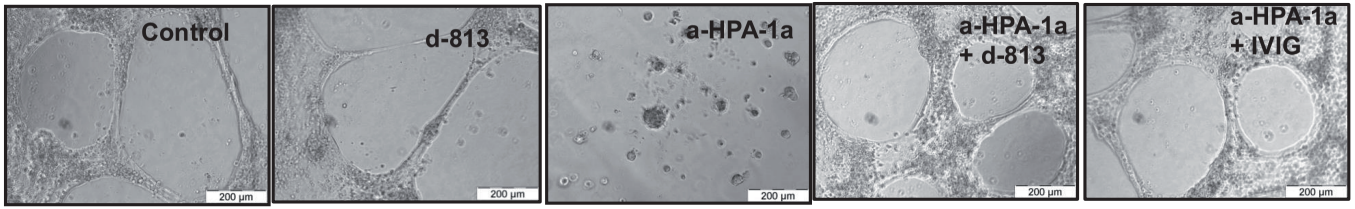
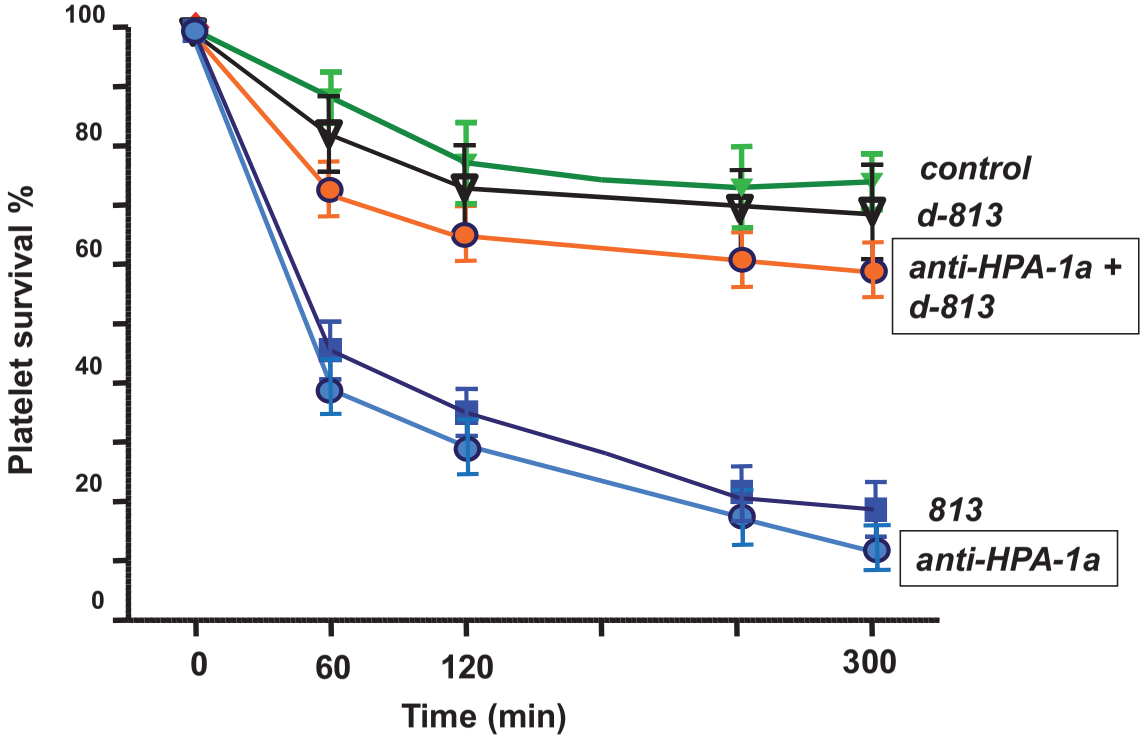

Control
d-813
a-HPA-1a
a-HPA-1a + d-813
a-HPA-1a + IVIG
200 µm

Figure 6


Platelet survival %
100
80
60
40
20
0
0
60
120
300
Time (min)
control
d-813
anti-HPA-1a +
d-813
813
anti-HPA-1a

14. The Fifth Publication (Attachment 5)

Alloantibody against new platelet alloantigen (Lap^a) on glycoprotein IIb is responsible for a case of fetal and neonatal alloimmune thrombocytopenia

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BACKGROUND: Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is caused by the destruction of platelets (PLTs) in the fetus or newborn by maternal PLT antibodies that crossed the placenta during pregnancy.

STUDY DESIGN AND METHODS: In this study, we aim to elucidate the properties of a new PLT alloantigen (Lap^a) that is associated with a severe case of FNAIT. Analysis of maternal serum with phenotyped PLTs by monoclonal antibody–specific immobilization of platelet antigens showed positive reaction against PLT glycoprotein (GP)IIb/IIIa and HLA Class I expressed on paternal PLTs.

RESULTS: In contrast to GPIIIa-reactive anti-HPA-1a, anti-Lap^a alloantibodies precipitated predominantly GPIIb. Indeed, a point mutation G>C at Position 2511 located in Exon 25 of the ITGA2B gene was found in Lap^a-positive donors. This mutation causes an amino exchange Gln>His at Position 806 located in the calf-2 domain of GPIIb. Lap^a-positive individuals were not found in 300 random blood donors. Our expression study showed that anti-Lap^a alloantibodies reacted with stable transfected HEK293 cells expressing the mutated GPIIb isoform (His806). CHO cells carrying this isoform, however, failed to react with anti-Lap^a alloantibodies, indicating that Lap^a epitopes depend on the Gln_{806}His mutation and the carbohydrate composition of the GPIIb. This mutation did not hamper the binding of anti-HPA-3a, which recognizes a point mutation (Ile_{843}Ser) located in calf-2 domain. Finally, we found that Lap^a and some HPA-3a epitopes are sensitive to O-glycanase.

CONCLUSIONS: This study not only underlines the relevance of rare HPAs on the pathomechanism of FNAIT, but also helps to understand the pitfalls of serologic assays to detect anti-GPIIb alloantibodies.

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is a severe bleeding disorder of the fetus and newborn, which is caused by destruction of platelets (PLTs) by maternal alloantibodies during the pregnancy and after birth. The alloantibodies are directed against human PLT antigen (HPA) expressed on fetal PLTs inherited from the father.[1] Six diallelic HPA systems (HPA-1, -2, -3, -4, -5, and -15) have been established. In addition, a number of private or rare HPAs associated with FNAIT have been reported. Meanwhile, 22 low-frequency HPAs (HPA-6bw to -14bw and HPA-16bw to -28bw) have been assigned. Most of them (n = 18) reside on glycoprotein (GP) IIb/IIIa, one on GPIbβ and three on GPIa (http://www.ebi.ac.uk/ipd/hpa/).

ABBREVIATIONS: FNAIT = fetal and neonatal alloimmune thrombocytopenia; GP(s) = glycoprotein(s); MAIPA = monoclonal antibody–specific immobilization of platelet antigens; SSP = sequence-specific priming.

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The GPIIb/IIIa complex (also known as $\alpha_{IIb}\beta_3$) is the major integral PLT GP that functions as receptor for fibrinogen and von Willebrand factor during PLT aggregation and adhesion.[2] The biosynthesis of GPIIb/IIIa in megakaryocytes is complex. Posttranslational processing including modification of carbohydrate moieties from high mannose to complex form, as well as proteolytic cleavage into heavy and light chains of GPIIb is important for the maturation and expression of intact GPIIb/IIIa complex on the PLT surface.[3] A conserved N-glycan at Position 15 seems to be important for the correct folding and complex formation of GPIIb/IIIa precursors.[4] In addition, it is also known that GPIIb carries an O-linked carbohydrates structure.[5] The functional role of this O-glycan in the biosynthesis of ITGA2B/B3, however, is unknown.

Molecular genetic studies have demonstrated that single-amino-acid mutations on PLT integrins are responsible for the formation of most HPAs.[6] However, several studies indicated that the binding sites of PLT alloantibodies depend not only on the single-amino-acid mutations, but also on the three-dimensional structure and posttranslational modification of the ITGA2B/B3 complex.[7-9] Furthermore, the binding sites of PLT alloantibodies seem to be heterogeneous; some PLT alloantibodies require an intact GPIIb/IIIa structure, whereas others do not.[10-12]

In this study, we describe a case of FNAIT caused by maternal alloimmunization against a low-frequency antigenic determinant on GPIIb (termed Lap[a]), which is not only formed by single missense mutation ($Gln_{806}Asn$), but also depends on the O-glycosylation of GPIIb.

CASE REPORT

The index patient was born as the second child of nonconsanguine parents. The sibling born 2 years before did not have any evidence of bleeding and has developed normally. The index patient developed hematoma and petechiae directly after birth. An initial full blood count showed a PLT count of 2×10^9/L (reference interval, $140 \times 10^9 - 360 \times 10^9$/L). The patient received a PLT transfusion with poor increment and required further transfusions for the next 2 weeks to maintain a PLT count of higher than 50×10^9/L. Initial serologic workup in a regional laboratory included a PLT antibody screen (GenProbe, Lifecodes Pak 12 enzyme-linked immunosorbent assay [ELISA], Immucor, Norcross, GA) as well as a PLT cross-match by indirect monoclonal antibody–specific immobilization of platelet antigens (MAIPA), both of which were reported as negative. We received a first sample 4 weeks after birth. The maternal serum was nonreactive in the indirect MAIPA with a panel of HPA-phenotyped PLTs except for weak reactions against HLA Class I. The cross-match test between maternal serum and paternal PLTs, in contrast, was clearly positive for GPIIb/IIIa and HLA Class I. Extended serologic testing did not reveal any known HPA specificity. Genotyping excluded incompatibility for rare HPAs residing on GPIIb/IIIa. The number of GPIIb/IIIa and GPIb/IX molecules expressed on the PLTs surface of the child was found to be normal (57,300 molecules/PLT and 36,000 molecules/PLT, respectively).

MATERIALS AND METHODS

Antibodies

Alloantibodies against HPA-1a and HPA-3a were obtained from a mother who gave birth to a child with FNAIT.[8,13] Control AB serum was obtained from a healthy male blood donor. Monoclonal antibodies (MoAbs) Gi5, Gi9, and Gi18 against GPIIb/IIIa, GPIa/IIa, and PECAM-1, respectively, were produced and characterized in our laboratory.[14] MoAb FMC25 against GPIb/IX complex was purchased from AbD Serotec (Oxford, UK). MoAb against β2-microglobulin associated with human HLA Class I (Clone B1G6) was from Beckman Coulter (Marseille, France), and MoAb TEA 2/16 against CD109 was purchased from BD Bioscience (Heidelberg, Germany).

Characterization of PLT alloantibodies by antigen capture assay

PLTs from the father and known HPA-phenotyped healthy blood donors were isolated from EDTA-anticoagulated blood by differential centrifugation and stored at 4°C in isotonic saline containing 0.1% NaN_3. Antibody detection was performed using antigen capture assay, MAIPA, using a panel of MoAbs against PLT GPs (see above) as previously described.[15]

Immunoprecipitation

PLTs and HEK293 cells (see below) were surface labeled with 5 mmol/L NHS-LC-Biotin (Pierce, Rockford, IL) and were precipitated as previously described.[16] Labeled cell lysates (100-300 μL) were incubated with 20 to 50 μL of serum or MoAb (20 μg/ml) overnight at 4°C in the presence of 50 μL of protein G beads (Gerbu Biotechnik, Heidelberg, Germany). After washings with immunoprecipitation buffer (50 mmol/L Tris, 150 mmol/L NaCl, 1% Triton X-100), bound proteins were eluted by adding sodium dodecyl sulfate (SDS) buffer for 5 minutes at 100°C. Eluates were analyzed on 7.5% SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions. Separated proteins were transferred onto polyvinylidene fluoride membranes and developed with peroxidase-labeled streptavidin and a chemiluminescence system (ECL, Amersham Biosciences, Glattbrugg, Switzerland).

Nucleotide sequencing analysis

Full-length sequencing of ITG2B and ITGB3 was carried out as described previously. Nucleotide sequences of polymerase chain reaction (PCR) primers, sequencing, and

reaction conditions are available upon request.[17] Briefly, GPIIb and GPIIIa coding regions of paternal genomic DNA was PCR amplified with primers corresponding to intronic sequence surrounding all exons of ITG2B and ITGB3. PCR was carried out using a PCR system (FastStart High Fidelity, Roche Diagnostic Corp., Indianapolis, IN). Before sequence analysis PCR products (ranged from 500-1200 bp) were purified with a PCR purification kit (QIAquick, Qiagen Sciences, Valencia, CA). Automated sequence analysis was performed in both directions on a genetic analyzer (ABI 3100, Applied Biosystems, Foster City, CA).

Genotyping by allele-specific PCR

Genomic DNA was extracted from peripheral blood leukocytes derived from 5 mL of EDTA anticoagulated by the use of a blood and tissue kit (DNeasy, Qiagen, Duesseldorf, Germany). One-hundred nanograms of genomic DNA was amplified using 1.25 μL (10 pmol/μL) of allele-specific forward primer (2492-5′-GCATCCACCTTCCGGGAC A(or G)-3′-2511) and common reverse primer (2622-5′-GATGGGCAGCCCCCAGTC-3′-2605), 4 μL of dNTP (1.25 mmol/L each nucleotide), 2.5 U of *Taq* Gold polymerase (Perkin-Elmer, Norwalk, CT), and 2.5 μL of buffer (10×) in a total volume of 25 μL. Thirty-four cycles of denaturation at 95°C for 30 seconds, annealing at 62°C for 30 seconds, and extension at 72°C for 30 seconds were performed. Before PCR, samples were denatured at 95°C for 5 minutes. HGH gene was run as internal control (forward primer 408-5′-CAGTGGCTTCCCAACCATTCCCTT-3′-432 and reverse primer 846-5′-ATCCACTCACG GATTTCT GTTGTGTTTC-3′-819). PCR products were analyzed by 2% agarose gel electrophoresis stained with ethidium bromide. A 100-bp DNA ladder standard was used (Biolabs, Ipswich, MA).

Construction of GPIIIa allelic expression vector

A full-length GPIIb cDNA in the mammalian vector pMPSV encoding for GPIIb His_{806} isoform (Lap^a) was produced by site-directed mutagenesis using a mutagenesis kit (Quick Change, Stratagene, Heidelberg, Germany) as previously described.[16] For PCR amplification, site-directed mutagenesis primers (5′-CCACCTTCCGGGACA CTCCC AGCCCTCCGAC-3′ and 5′-GTCGGAGGGCTGGGA GTGTCCCGGAGGAAGGTGG-3′) encompassing nucleotides (2496-2526) of GPIIb cDNA were constructed. After denaturation for 30 seconds at 95°C, GPIIb wild-type plasmid (20 ng) was amplified for 12 cycles (denaturation at 95°C for 30 sec, annealing at 55°C for 1 min, and extension at 65°C for 12 min). PCR products were digested with *Dpn*I endonuclease for 1 hour at 37°C and transformed into DH5α high-efficiency competent *Escherichia coli* bacteria (Invitrogen, Carlsbad, CA). Plasmid DNA from positive clones was verified by nucleotide sequencing using reverse primer (2622-5′-GATGGGCAGCCCCCAGTC -3′-2605) and forward primer (5′-2418-CTGGGGACCCAA AGTGGAGC-3′-2437) as described.

Stable transfection of Lap^a alloantigen in CHO and HEK cells

CHO cells (American Type Tissue Collection, Rockville, MD) cells were grown in α-MEM (PAN, Aidenbach, Germany) supplemented with 10% fetal calf serum (FCS; PAN) and were transfected with allele-specific GPIIb constructs encoding for Gln_{806} (wild type) or His_{806} (mutant) isoform together with wild-type GPIIIa construct as previously described.[16] HEK293 cells (American Type Tissue Collection) were grown in DMEM containing 10% FCS and 1% penicillin-streptomycin and were transfected with GPIIb allelic constructs and GPIIIa wild-type plasmid using Superfect as recommended by the manufacturer (Qiagen, Hilden, Germany). Stably expressing cells were selected with genicitin and zeocin (genicitin final concentration 800 μg/mL; zeocin 250 μg/mL; GIBCO BRL, Grand Island, NY) and were sorted by the use of fluorescein isothiocyanate (FITC)-labeled MoAb against CD41 (Becton Dickinson, Heidelberg, Germany) by flow cytometry (FACSAria, Becton Dickinson).

Flow cytometric analysis of stably transfected CHO and HEK293 cells

Transfected cells were cultured over 2 weeks, and the surface expression of recombinant GPIIb/IIIa complex was measured by flow cytometry (FACSCalibur, Becton Dickinson) as previously described.[16] Cells were incubated with 20 μL of serum for 30 minutes at 4°C or with 20 μL of FITC-labeled MoAb against CD41 (Becton Dickinson). After being washed with phosphate-buffered saline (PBS) buffer containing 0.5% bovine serum albumin, cells were labeled with FITC-conjugated anti-human IgG antibody (dilution 1:50; Dako, Hamburg, Germany), washed, and measured as described.

Deglycosylation of GPIIb/IIIa on PLTs and transfected HEK cells

A total of 2×10^6 PLTs or 5×10^5 transfected HEK293 cells were treated with 1 μL of O-glycanase (75.000 units, New England Biolabs, Ipswich, MA) for 2 hours at 37°C in a thermomixer (Eppendorf, Hamburg, Germany). After incubation, cells were washed two times with 100 μL of PBS buffer. One-hundred microliters of treated and untreated PLTs or HEK293 cells was analyzed by the MAIPA assay as described above.

RESULTS

Serologic and immunochemical analysis

The cross-match analysis between maternal serum and paternal PLTs in the MAIPA assay showed clear reactions when MoAbs against GPIIb/IIIa and HLA Class I were used as capture antibodies, but not with MoAbs against GPIb/IX, GPIa/IIa, PECAM-1 and CD109 (Fig. 1A). When maternal serum was tested with a panel of HPA-phenotyped PLTs (12 different PLT suspensions), no reaction was observed (data not shown). Furthermore, extended genotyping of GPIIb and GPIIIa polymorphisms ruled out the presence of known rare HPAs on paternal PLTs. These results indicated that the maternal serum contained an alloantibody against a new low-frequency PLT alloantigen on GPIIb/IIIa, which we termed Lap^a. When immunoprecipitation analysis was performed with biotin-labeled paternal PLTs, maternal serum precipitated predominantly PLT GPIIb (Fig. 1B). This band could not be precipitated from PLTs of a normal donor.

Genetic analysis

To ascertain the molecular genetic basis underlying the Lap^a antigen, paternal genomic DNA corresponding with the coding regions of ITGA2B and ITGB3 was sequentially amplified by PCR using 28 sets of primers. No point mutation was found in ITGB3 gene (data not shown). However, nucleotide sequencing of ITGA2B gene encompassing the full length of GPIIb transcript showed one nucleotide substitution G>C at Nucleotide 2511 located on Exon 25 of the ITGA2B gene (Fig. 2A). This mutation predicted the amino acid Gln (negatively charged side group) at Position 806 in Lap^a-negative and His (positively charged side group) in Lap^a-positive individuals. This result was confirmed by nucleotide sequence analysis of the child (data not shown). Alignment analysis between human, mouse, and canine genes showed that this mutation occurred in the calf-2 domain of GPIIb.

Genotyping of family member by PCR–sequence-specific priming

To study the paternal inheritance of Lap^a, genotyping based on allele-specific PCR was established. Allele-specific forward primers located in Exon 25 and universal primer in Exon 26 were designed to amplify a 247-bp PCR product (Fig. 2A). Figure 2B shows the pedigree of the Lap^a family. In accordance with the nucleotide sequencing results, the index neonate (III.2) as well as the father (II.2) were typed as heterozygous Lap^a, whereas the mother (II.1) was Lap^a negative. Among 10 siblings, only four DNA samples could be obtained. Two (II.3 and II.8) were heterozygous Lap^a, and two were negative (II.4 and II.7). This result demonstrates the codominant inheritance of

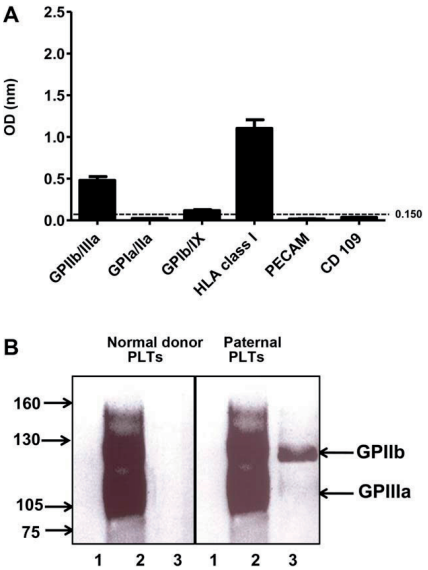

Fig. 1. (A) Serologic analysis of Lap^a antibody by MAIPA. Paternal PLTs were incubated with maternal serum together with MoAbs against PLT GPs (anti-GPIIb/IIIa, -GPIa/IIa, -GPIb/IX, -PECAM, -CD109, and -HLA Class I). Bound human antibodies were then detected by the use of enzyme-labeled secondary antibody and substrate system. Reaction was measured at OD490/620 using ELISA microtiter reader. (B) Immunoprecipitation analysis of anti-Lap^a. Paternal and control PLTs were surface labeled with biotin and lysed. Labeled PLT lysates were precipitated with control serum (Lanes 1), anti-HPA-1a (Lanes 2), and maternal serum (Lanes 3). Immunoprecipitates were run on 7.5% SDS-PAGE under reducing conditions. Separated proteins were then transferred onto polyvinylidene fluoride membrane and visualized by the use of streptavidin and a chemiluminescence system.

Lap^a antigen in the index family. However, further analysis by using PCR–sequence-specific priming (SSP) in 300 unrelated Caucasian blood donors did not find any Lap^a-positive individuals.

Expression study of Lap^a alloantigenic determinant on mammalian cells

Allele-specific constructs encoding wild-type GPIIb (Gln_{806}) or mutant GPIIb (His_{806}) were transfected into hamster CHO cells together with GPIIIa wild-type construct to prove the role of $Gln_{806}His$ mutation on the

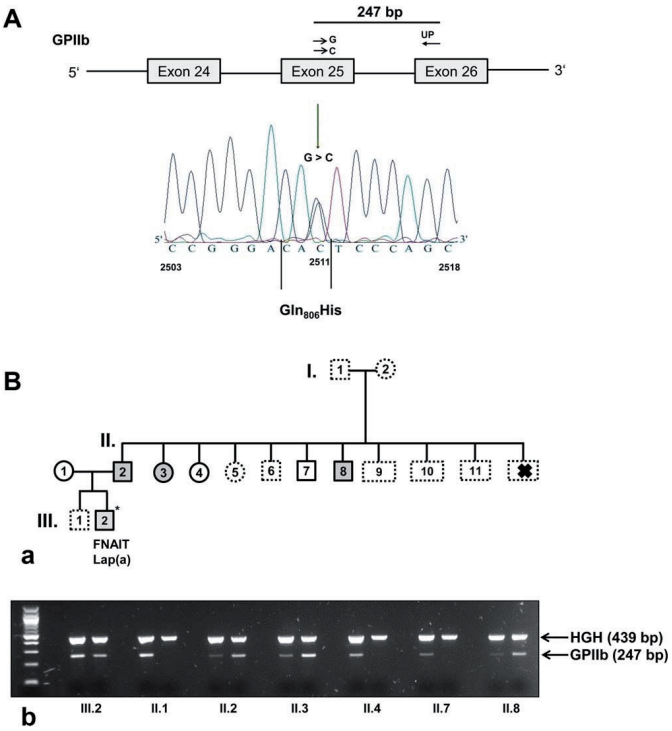

Fig. 2. (A) Nucleotide sequencing analysis of amplified GPIIb from paternal DNA. Sequencing of PCR product encompassing nucleotides 2503 to 2518 of GPIIb gene is presented. The nucleotide exchange G>C at Position 2511 (in heterozygous state) in Exon 25 results in Gln_{806} (CAG) > His_{806} (CAG) is shown. The positions of allele-specific primers used for PCR-SSP are indicated. (B) Pedigree and genotyping of the Lapa family. Genomic DNA was isolated from the family member and was amplified by PCR-SSP (see above) using human growth hormone (HGH) as internal control (439 bp). PCR products (247 bp) were visualized on 2% agarose gel electrophoresis. Open symbols = Lap^a-negative individuals; solid symbols = Lap^a-positive individuals. Dotted lines = not done; × = dead.

formation of Lap^a alloantigen. Surprisingly, CHO cells transfected with mutant GPIIb construct did not show any reaction with anti-Lap^a alloantibody in flow cytometry (Fig. 3, left panel), although intact GPIIb/IIIa could be detected by the use of complex-dependent MoAb Gi5. To exclude the role of posttranslational modification of GPIIb/IIIa on different cell lines, human-derived HEK293 cells were transfected with GPIIb constructs as above. In contrast to the results that were obtained with CHO transfectants, stable transfected HEK cells expressing mutant GPIIb/IIIa showed positive reaction with anti-Lap^a alloantibody (Fig. 3, right panel). In the control experiment, no reaction was found with anti-Lap^a and the wild-type GPIIb/IIIa. Similar results were obtained by immunoprecipitation analysis (data not shown). These results indicated that Lap^a alloantigenic determinants not only depend on the point mutation Gln_{806}His, but also on the posttranslational modification of GPIIb. Furthermore, study was performed to prove whether some MoAb used as capture antibody may inhibit the binding of anti-Lap(a) antibodies leading to false-negative reaction or weak reactivity. Weak reactivity was obtained when MoAb SZ21 and MoAb SZ22 against GPIIIa and GPIIb, respectively, were used. This result indicated that MoAb against GPIIb/IIIa complex is advantageous for the detection of anti-Lap(a) antibody (Fig. S1, available

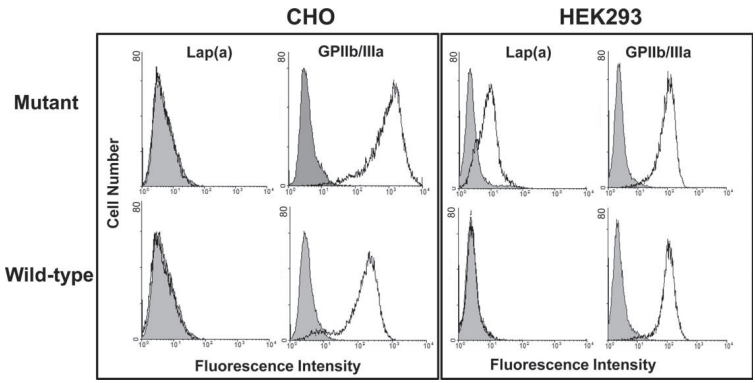

Fig. 3. Flow cytometry analysis of cell-expressing mutant and wild-type GPIIb/IIIa. CHO- and HEK293-transfected cells were incubated with MoAb against GPIIb/IIIa (Clone Gi5) or anti-Lap^a. Isotype mouse IgG and AB serum were run as controls in parallel. After being washed, cells were labeled by secondary antibody (Alexa Fluor–labeled donkey anti-mouse IgG or fluorescein-conjugated rabbit anti-human IgG). Labeled cells were analyzed by flow cytometry. Smooth lines = negative controls; bold lines = anti-GPIIb/IIIa and anti-Lap^a reactivity.

as supporting information in the online version of this paper).

Figure 4A shows the location of Lap^a in the calf-2 domain of GPIIb and three putative O-glycosylation sites, one located adjacent, only five amino acids upstream (at S801) of the polymorphic residue 806. Two further O-glycosylation sites (at S845 and S847) are located more closely to the Ile843Ser mutation associated with the HPA-3 alloantigen system. To prove the role of O-glycosylation on the expression of Lap(a) antigen, HEK-transfected cells were treated with O-glycosidase. Nontreated and treated cells were then tested in the MAIPA assay using MoAb Gi5 as capture antibody (Fig. 4B, left panel). When transfected cells were treated with O-glycosidase significant reduction of anti-Lap^a binding was detected. In contrast, no change of anti-HPA-1a binding was observed upon treatment of transfectants with this enzyme. In the control experiment anti-Lap^a alloantibody did not react with wild-type GPIIb/IIIa. Similar results were obtained with PLTs. Fresh Lap^a-positive PLTs lost their reactivity against anti-Lap^a antibodies after treatment with O-glycosidase (Fig. 4B, right panel). This result indicated that O-glycan contributes to the generation of Lap^a alloantigenic determinants.

Reactivity of anti-Lap^a with HEK-transfected cells after a long culture period

To study the stability of Lap^a for a period of time, HEK293-transfected cells expressing Lap^a alloantigenic determinant were cultured for 6 weeks, and cells were analyzed every week by flow cytometry (Fig. 5). A continuous reduction of anti-Lap^a alloantibody binding was observed every week. After 5 weeks, cells no longer showed any reaction with anti-Lap^a alloantibody (mean fluorescence intensity [MFI], 17.33 vs. 2.59). In contrast, the expression of GPIIb/IIIa on the cell surface of the cells did not change significantly within this period (MFI, 157.84 vs. 126.98), and to prove the possibility that cell freezing may cause the reduction of Lap(a) antigen rather than culture conditions, transfected cells were thawed after 4 months of storage in liquid nitrogen and were tested by the MAIPA assay as previously described. No significant change was detected indicating that freezing-thawing procedure did not alter the expression of Lap(a) antigenic determinants (Fig. S1, available as supporting information in the online version of this paper). Although our observation indicated that PLTs or transfected cells stored for a short period (2-5 weeks) could be used for the detection of anti-Lap(a) antibodies in the MAIPA, the use of fresh PLTs, transfected cells, or fresh thawed cells is recommended.

Two different types of anti-HPA-3a

In our previous study, we found that some anti-HPA-3a alloantibodies reacted with recombinant HPA-3a expressed on CHO cells (Type I) and some failed to react with these transfected cells (Type II).[12] Figure 6 shows the reactivity of Type I and Type II anti-HPA-3a alloantibodies with HPA-3a expressed either on CHO or by HEK293 cells (wild type). Whereas Type I HPA-3a antibodies reacted with both transfected cells, Type II antibodies only

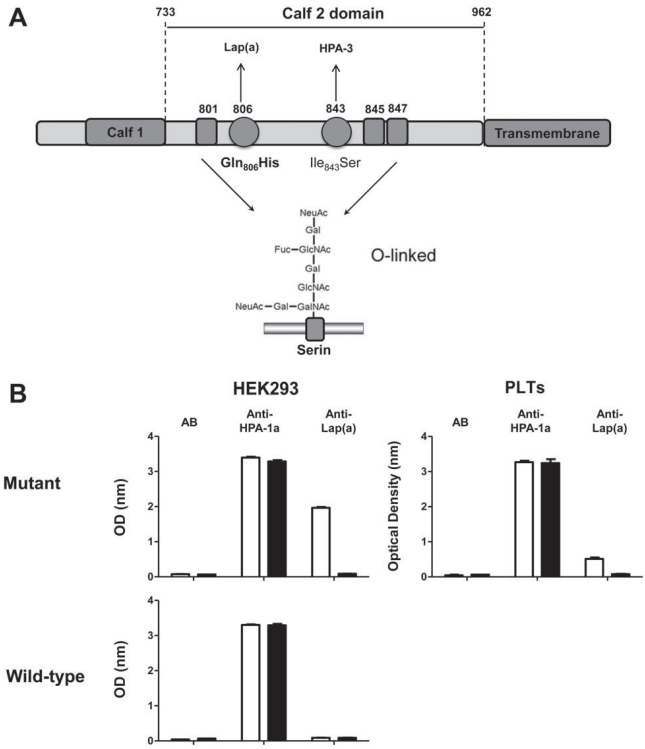

Fig. 4. (A) Location of Gln806His associated with Lap[a] mutation in the calf-2 domain of GPIIb. The positions of three putative O-linked glycosylation sites (at Amino Acids S_{801}, S_{845}, and S_{847}) and $Ile_{843}Ser$ mutation responsible for HPA-3 system are indicated. (B) MAIPA analysis of Lap[a]-positive cells treated with O-glycosidase. HEK293-transfected cells and PLTs expressing Lap[a] antigen were treated with O-glycosidase or PBS (as control). After being washed, cells were incubated with anti-HPA-1a, anti-Lap[a], and AB serum as negative control together with MoAb Gi5 as capture antibody. After lysis, cells that bound human antibody were detected using enzyme-labeled goat anti-human IgG and were analyzed by spectrophotometry. Reaction was measured at OD490/620 using an ELISA microtiter reader. (□) Untreated cells; (■) O-glycosidase–treated cells.

reacted with HPA-3a expressed on HEK293 cells, but not on CHO cells. However, this reaction disappeared when HEK cells were treated with O-glycosidase (Fig. 6, right panel), similar to the phenomenon observed with anti-Lap[a] antibodies (see Fig. 4B). In contrast, Type I anti-HPA-3a still reacted with O-glycosidase–treated cells. Finally, we tested our transfected HEK cells expressing Lap[a] as well as HPA-3a (mutant) with different anti-HPA-3a by flow cytometry. All anti-HPA-3a (n = 4) tested showed a positive reaction with these cells, indicating that $Gln_{806}His$ mutation did not alter the binding of anti-HPA-3a (data not shown).

DISCUSSION

In this study, we report on a new rare alloantigen, Lap[a], located on PLT GPIIb, involved in a case of FNAIT. Indeed this serum contained antibodies against HLA Class I molecules. Although anti-HLA Class I antibodies

are often detectable in pregnant women, their role in NAIT is considered controversial. Husebekk and Skogen[18] reported 51 of 195 cases of children with thrombocytopenia in whom only anti-HLA Class I antibodies were detected, indicating that anti-HLA Class I antibodies may cause thrombocytopenia in the fetus and newborn. However, the mechanism is not yet formally demonstrated.

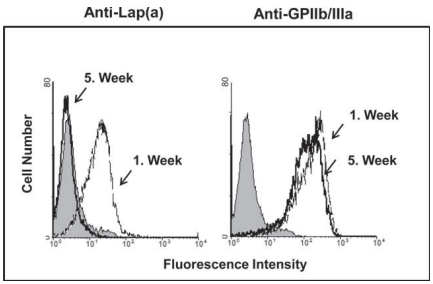

Fig. 5. The stability Lap[a] antigen expressed on HEK293 cells after a long culture period. HEK293-transfected cells were incubated by MoAb Gi5 or anti-Lap[a]. Isotype mouse IgG and AB serum were run as controls in parallel. After being washed, cells were labeled by Alexa Fluor donkey anti-mouse IgG or fluorescein-conjugated rabbit anti-human IgG. Labeled cells were analyzed by flow cytometry.

Examination of the nucleotide sequence of ITGA2B derived from the Lap[a]-positive father showed one nucleotide substitution G>C at Position 2511 in the heterozygous state located in Exon 25. Whereas Lap[a]-negative individuals carry glutamine at Position 806, Lap[a]-positive individuals bear histidine at this position. In a population study, none of 300 unrelated donors was found to carry the Lap[a] alloantigen. Analysis of recombinant allele-specific GPIIb/IIIa in human mammalian cells (HEK293 cells) showed that the single-amino-acid substitution $Gln_{806}His$ is directly responsible for the formation of Lap[a] alloantigenic determinant(s). However, we found evidence that glycosylation of GPIIb is required for the proper expression of this new PLT alloantigen since anti-Lap[a] was nonreactive 1) when the antigen was expressed in CHO cells, 2) when sugars were removed from transfected HEK cells by glycosidase treatment, and 3) when transfected HEK cells were cultured for a longer period of time.

The fact that glycosylation patterns differ between proteins synthesized in HEK293 and CHO cells is known; in

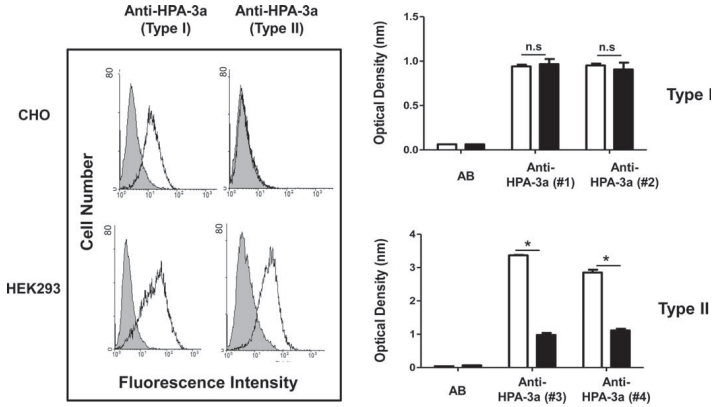

Fig. 6. (A) The reactivity of different anti-HPA-3a with HPA-3a–transfected cells. CHO- or HEK293-transfected cells expressing HPA-3a were incubated with different anti-HPA-3a (Type I and Type II) containing sera or AB serum as control. After washings, bound antibodies were labeled with fluorescein-conjugated rabbit anti-human IgG and were analyzed by flow cytometry. Smooth line = negative control; solid line = anti-HPA-3a. Note: Type I anti-HPA-3a reacted with both CHO- and HEK-transfected cells. Type II anti-HPA-3a reacted only with HEK-transfected cells. (B) MAIPA analysis of HPA-3a–positive HEK293 cells treated with O-glycosidase. HEK293-transfected cells expressing HPA-3a antigen were treated with O-glycosidase (■) or PBS (□). After being washed, cells were incubated with Type I (#1, #2) and Type II anti-HPA-3a (#3, #4). MoAb Gi5 that reacted with GPIIb/IIIa complex was used as capture antibody. After lysis, cells that bound human antibody were detected using enzyme-labeled goat anti-human IgG and were analyzed by spectrophotometry. Reaction was measured at OD490/620 using an ELISA microtiter reader. *Significant for $p < 0.05$ ($p = 0.009$); n.s. = not significant for $p < 0.05$ ($p = 0.9893$).

comparison to CHO cells, HEK293 mammalian cells are able to synthesis N- and O-glycosylation structures of higher complexity.[19] The point mutation $Gln_{806}His$ associated with Lap[a] is located in the calf-2 domain of GPIIb, which contains three putative O-glycosylation sites (Ser_{801}, Ser_{845}, and Ser_{847}), one of which, Ser_{847}, was identified as the actual site of O-glycosylation.[5,20] This physical proximity is a potential explanation for the involvement of glycosylation in the formation of the Lap[a] epitope(s). Treatment of HEK293 cells expressing Lap[a] with O-glycosidase abolished the binding of anti-Lap[a], supporting the concept that a correct O-glycosylation of GPIIb is involved in Lap[a] epitope(s) formation. The question whether O-linked carbohydrates attached to Ser_{847} or to other serine residues (Ser_{845} or Ser_{806}) is directly involved in the Lap[a] antigen formation, however, remains to be established.[20]

When transfected HEK cells were cultured for approximately 6 weeks and tested every week for surface expression by flow cytometry, no change of GPIIb/IIIa surface expression was observed. In contrast, the expression of the Lap[a] antigen decreased continuously and disappeared in Week 6. A recent study demonstrated that changing culture medium and conditions could alter glycosylation processes of different proteins in HEK cells,[19] again underlining the importance of correct GPIIb glycosylation for the Lap[a] antigen formation.

In our previous study, we observed that some anti-HPA-3a (six of 10) reacted with HPA-3a expressed on CHO cells.[12] The HPA-3a alloantigen is not only determined by a single-amino-acid mutation, $Ile_{843}Ser$, but also depends on the O-glycosylation of Ser_{847}[5,8] and sialic acid residues.[7,12,21] When we retested anti-HPA-3a that failed to react with CHO cells, positive reactions were obtained with HPA-3a expressed on HEK cells. Furthermore, this reactivity was destroyed when HEK cells were treated with O-glycosidase before incubation with anti-HPA-3a. These results indicate that two different types of anti-HPA-3a may develop during alloimmunization: Type 1, which recognize carbohydrate-independent epitopes (reactive with HPA-3a expressed on CHO cells and with deglycosylated HPA-3a expressed on HEK cells), and Type 2, which recognize carbohydrate-dependent epitopes (nonreactive with HPA-3a expressed on CHO cells and nonreactive with deglycosylated HPA-3a expressed on HEK cells).

This is a relevant observation because allele-specific transfected cell lines expressing low-frequency HPAs have been introduced as PLT substitutes for the detection of PLT alloantibodies.[22,23] Based on the fact that glycosylation patterns may differ and even change during cell culture, screening for PLT antibodies with such cell lines requires careful validation.

Taken together, we have demonstrated once more the relevance of rare HPAs residing on PLT GPIIb/IIIa in the pathomechanism of FNAIT and the importance of performing serologic cross-matches between maternal serum and paternal PLTs in the workup of thrombocytopenic neonates. Furthermore, we underline the contribution of carbohydrates on the formation of Lap[a] and HPA-3a epitopes and confirm the existence of different HPA-3a antigen-antibody types. This knowledge may help us to design better serologic assays to detect PLT-reactive antibodies against GPIIb. Furthermore, the question whether some PLT antibody types reacted with different glycosylated GPIIb may impair PLT clearance or PLT function is intriguing.

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1. *Top*: Analysis of wild type (white columns) and mutant transfected cells (black columns) after 4 months storage in liquid nitrogen with AB serum, anti-HPA-1a and anti-Lap(a) antibody by the MAIPA using MoAb Gi5 as capture antibody. Note: positive reaction of Lap(a) antibody with mutant cells could be detected although the cells were already kept for few months in liquid nitrogen. *Bottom*: Analysis of anti-Lap(a) antibody with mutant cells by the MAIPA assay using MoAbs against GPIIb/IIIa complex (clone Gi5), GPIIIa (clone SZ21) and GPIIb (clone SZ22). Note: strong reaction was only obtained with GPIIb/IIIa specific MoAb.

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