Molecular genetic analysis of patients with rare bleeding disorders in South Iran

Summary & Conclusion

Hereditary platelet function defects such as Bernard-Soulier syndrome (BSS) and Glanzmann thrombasthenia (GT) affect primary hemostasis and lead to bleeding diathesis.

BSS is a recessively inherited hemorrhagic disorder caused by the quantitative or functional deficiency of one of the glycoproteins forming the GPIbα/Ibβ/IX/V complex. The platelet receptor for the von Willberand factor on the platelet surface, in patients affected by BSS platelet adhesion to the damaged vascular wall, is severely impaired but platelet aggregation and secondary hemostasis are normal (chapter 2.2).

GT is a recessively inherited bleeding disorder that is caused by the quantitative or functional deficiency of integrin αIIbβ3 (or glycoprotein IIb/IIIa), which is the platelet fibrinogen receptor (chapter 2.4).

GT and BSS are extremely rare disorders. Our findings are remarkable for the high prevalence of GT and BSS relative to the general population of South Iran. Assuming that we were able to identify all the affected patients, an approximate prevalence of 1:200 000 for GT and 1:600 000 for BSS can be estimated (chapter 2.1).

We reviewed the clinic and hospital records of 40 patients before and after referral to our specialized center in order to describe the natural history of these conditions in terms of bleeding symptoms and transfusion requirements (chapter 2.1). Twenty-three patients from 16 different families were diagnosed with GT. Consanguinity among parents was present in all but one patient. Diagnosis was made in patients who had a normal platelet count, severely impaired platelet aggregation with high concentrations of ADP, collagen and arachidonic acid but normal aggregation with ristocetin. On flow cytometry, 19 patients were diagnosed type I GT (<5% of GpIIb/IIIa expression) and four type II GT (GpIIb/IIIa expression between 5% -20%). Median age at first symptoms was 2 years; >50% of patients were diagnosed because of epistaxis.

BSS was diagnosed in seven individuals from four different families and all patients had consanguineous parents. Diagnosis was made on patients who had normal platelet aggregation to ADP, collagen and arachidonic acid but a severely defective response to large concentrations of ristocetin (1.5 mg/ml). Flow cytometry revealed that GpIb

expression was <2% in six cases compared with the expression in a control, in the remaining patient it was 12%. In these patients, platelet count was always low. When blood smears were analyzed macrothrombocytopenia was a constant feature.

The morbidity associated with GT was significant, because two-thirds of patients came to our observation due to bleeding complications before the age of five and >80% of the patients with GT were transfused at least once to control bleeding. Moreover, in this group of patients the median hemoglobin level was 10.3 g/dl, probably indicative of the presence of unadverted chronic bleeding. The vast majority of GT patients were type I, only four patients being diagnosed with type II. No patient had type III (chapter 2.1).

BSS was associated with significant bleeding problems that required medical attention early in life, Median age at first symptom was 15 months; the most common presentations were post injection or post vaccination. Three patients had been transfused more than five times, two at least once, while the remaining two had not required platelet transfusion (chapter 2.1).

In conclusion, we have reported the first complete laboratory-based investigation of a large cohort of South Iranian patients with GT or BSS, showing that both of these disorders are associated with significant bleeding diathesis (chapter 2.1). Earlier diagnosis of these patients would be helpful for their clinical management; therefore, these diagnoses should be aggressively sought in the offspring of consanguineous marriages presenting with a suggestive bleeding history.

We sequenced the GPIb and GPIX genes of the seven BSS patients and of all the available first and second degree relatives, in order to identify the mutations causing BSS. The presence or the absence of bleeding symptoms, and GPIb/IX/V expression were investigated in heterozygous carriers identified through genotyping. In two of the three families analyzed we found a missense mutation (a GpIX Phe 55Ser substitution) previously described in two patients, one from Japan and one from The Netherlands (chapter 2.2). The mutation is located in the leucine-rich motif of GpIX, in an area that is critical for GpIX-GpIb association. It is well known that only if all the subunits of the glycoprotein complex associate in the correct fashion can GpIb/IX/V be expressed on the plasma membrane of platelets. In one family we identified a previously undescribed

cytosine insertion at position 3221 causing a frame shift of the entire GpIb alpha gene, and it is reasonable to expect that no protein is produced. Actually, no GpIb/IX/Vwas detected by flow cytometry on platelets of affected individuals. The second purpose of this study was to investigate, in terms of bleeding symptoms, platelet number, morphology and GpIb/IX/V surface expression carriers and normal individuals in the same families.

No reduction in platelet count or signs of macrothrombocytopenia were found in two families carrying the same BSS mutation. In one family, a slight reduction in GpIb expression in heterozygous carriers was observed by flow cytometry, while in one family expression was above 75% in all individuals tested. The addition of the GpIb alpha nucleotide substitution GPIBA 3064 T>C ,a polymorphism known to enhance GpIb expression, probably contributes to normalize the complex expression in heterozygous carriers (chapter 2.2).

Analyzing the mutations of Italian GT patients we found that a patient had a αIIb G236E missense substitution that substitutes glycine from the highly conserved motif of blade 4 of the β-propeller. In this study (chapter 2.4), the effects of a mutation causing GT were analyzed using flow cytometry, biochemistry and immunofluorescence. The mutation was chosen because it causes the loss of a predictably very important glycine from the αIIb-β A propeller, since it is inserted between two rings of aromatic residues that stabilize the αIIb-β3 interface. Our hypothesis was that the mutation alters the association surface of the αIIb-β A propeller and destabilizes the complex with β3.

We showed by flow cytometry analysis that transfected cells express at very low levels the mutant αIIbβ3 on their membrane, a finding compatible with the GT phenotype.

We demonstrated that αIIbG236E is produced at levels comparable to those of normal αIIb, but several aspects of its maturation/association process are defective. The association with β3 in the endoplasmic reticulum is indeed hampered, and this is demonstrated by the presence of an increased amount of pro-αIIbG236E in transfected cells. Moreover, in Western blots from lysates of cells transfected with αIIbG236E, a band of low molecular weight reacting with antibodies against αIIb was identified, probably a sign of degradation of the uncoupled mutated protein. Even though a certain amount of the complex is formed and moves to the Golgi (as demonstrated by immunofluorescence studies) αIIbG236E and β3 appear to loosely associated, as shown by the inability of an anti-αIIb antibody to

immunoprecipitate β3 in lysates from transfected cells.

In the present study, we confirmed that a critical area for the αIIb-β3 association is the aromatic residues' rings, and inserting residues that either alter charge or that are bulky interferes with the biogenesis of the receptor, thereby giving rise to GT (chapter 2.4).

Factor X (FX) deficiency is a rare severe hemorrhagic condition inherited as an autosomal recessive trait. It is one of the most severe recessive inherited coagulation disorders. We analyzed the clinical manifestations, laboratory phenotype and genotype in 10 patients with severe FX deficiency and in their heterozygous relatives (chapter 2.3). The most frequent bleeding episodes were hematomas (70%) and gum bleeding (60%). In all, 50% of the homozygous patients required blood transfusion and one-third of heterozygotes required treatment after surgery or delivery. Homozygous candidate mutations were identified, and the genetic characterization revealed six different missense mutations, two of which were novel. The first mutation was p.Glu69Lys, consisting of a G to A transition (c.205G>A) in exon 2 encoding the Gla-domain, identified in an 11 years old boy. The vitamin K-dependent g-glutamyl carboxylase catalyzes the post-translational modification of specific glutamic acid residues to g-carboxyglutamic acid (Gla) residues in the Gla-domain.

The second novel mutation was p.Asp103His, identified in a 19 years old woman, consisting of a G to C transversion (c.307G>C) in exon 4 encoding the EGF1 domain. Correct post-translational modifications, such as g-carboxylation, glycosylation and b-hydroxylation, are essential for the FX protein to be fully functional, because they may alter physical and chemical properties, including folding and thus protein stability (chapter 2.3). The b-hydroxylation site of FX is located in the first EGF domain at Asp103, resulting in a b-hydroxyaspartic acid. The replacement of histidine at this position will probably result in the loss of FX function by affecting b-hydroxylation and thereby causing a severe FX deficiency. The remaining four mutations had been reported and were all identified in the homozygous state.