The molecular genetic basis of Glanzmann thrombasthenia in the Iraqi-Jewish and Arab populations in Israel

(platelet disorders/molecular genetics/glycoprotein IIb-IIIa/polymerase chain reaction)

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ABSTRACT Glanzmann thrombasthenia is an autosomal recessive bleeding disorder characterized by a decrease or absence of functional platelet glycoprotein (GP) IIb-IIIa ($\alpha_{IIb}\beta_3$) integrin receptors. Although thrombasthenia is a rare disorder, its occurrence is increased in some regions of the world where intracommunity marriage and consanguinity are commonplace, resulting in increased expression of autosomal recessive traits. We have been studying two populations having an unusually high frequency of Glanzmann disease, Iraqi Jews and Arabs living in Israel, and were able to distinguish the populations on the basis of immunodetectable GPIIIa and platelet surface vitronectin receptor ($\alpha_v\beta_3$) expression. In this article, we describe molecular genetic studies based on use of the PCR that have allowed us to characterize platelet mRNA sequences encoding GPIIb and GPIIIa from patients in these populations. In six of six Iraqi-Jewish families studied, cDNA sequence analysis identified an 11-base deletion within exon 12 of the GPIIIa gene. This mutation produces a frameshift leading to protein termination shortly before the transmembrane domain of GPIIIa. In contrast, a 13-base deletion encompassing the splice acceptor site of exon 4 of the GPIIb gene was found in three of five Arab kindreds studied. This deletion results in forced alternative splicing to a downstream AG acceptor, producing a 6-amino acid deletion in the GPIIb protein, including a single cysteine residue. These nucleotide sequence variations were exploited to design a rapid, PCR-based oligonucleotide dot-blot hybridization test for both pre- and postnatal diagnosis of Glanzmann disease. These studies demonstrate the heterogeneity of Glanzmann thrombasthenia in different populations, and its homogeneity within geographically restricted populations, and offer insight into the requirements for integrin surface expression.

Glanzmann thrombasthenia is a congenital bleeding disorder defined by defective or quantitatively abnormal platelet glycoprotein (GP) IIb-IIIa ($\alpha_{IIb}\beta_3$) receptors (1–3). The diagnosis of this disorder is based on a clinical syndrome of mucocutaneous bleeding in association with abnormal platelet aggregation in response to physiologic stimuli (e.g., ADP, thrombin, collagen, and epinephrine), normal platelet counts, and prolonged bleeding times. Recent biochemical and molecular biologic characterization of the GPIIb and GPIIIa integrin subunits (4–7), as well as studies on the biosynthesis of the GPIIb-IIIa complex (8–11), have shed considerable light on the possible etiologies of Glanzmann thrombasthenia. In particular, it is known that GPIIb and GPIIIa are the products of separate genes, and data indicate that GPIIb and GPIIIa must form a complex after synthesis in order to undergo final processing (including cleavage of GPIIb into a two-chain form) and transport to the platelet membrane (12).

Furthermore, since uncomplexed GPIIb and GPIIIa appear to undergo rapid catabolism (12), it is theoretically possible for a defect in either protein to lead to a deficiency in the expression of both subunits. Characterization of thrombasthenic platelet glycoprotein content by sensitive immunoblot (13–14) and flow cytometric techniques (15) has revealed that platelets of some thrombasthenics contain residual amounts of GPIIIa, while others contain small amounts of GPIIb, providing evidence that Glanzmann thrombasthenia is likely to be a heterogeneous disorder at both the protein and genetic levels. Interestingly, in spite of the likely heterogeneity in the molecular nature of the disease, the severity of bleeding in thrombasthenia does not seem to correlate with the qualitative or quantitative nature of the observed glycoprotein defect (3).

Glanzmann thrombasthenia is a relatively common disorder in the Iraqi-Jewish population in Israel, with 39 patients from 19 kindreds identified (16). The Iraqi-Jewish population has existed as a cohesive community since 586 B.C. with intragroup and consanguineous marriage common. Consanguinity is also common within the Arab population in Israel, where five affected Arab kindreds with 13 patients have been studied. The patients from both groups have <5% GPIIb-GPIIIa content, have absent clot retraction, and contain little or no intracellular platelet fibrinogen (16, 17). Previous studies have differentiated the Iraqi-Jewish thrombasthenics from their Arab counterparts on the basis of GPIIIa content—whereas 1–5% GPIIIa is detectable in Arab patients by immunoblot analysis, GPIIIa is undetectable in platelets from Iraqi-Jewish thrombasthenics. In contrast, a residual amount of GPIIb is present within patient platelets from both groups, and at least some of the GPIIb appears to be in the unprocessed, pro-GPIIb state (18). In addition, we have recently obtained data indicating that platelets from the Iraqi-Jewish patients also lack the vitronectin receptor ($\alpha_v\beta_3$), whereas platelets from Arab patients express either normal or increased numbers of vitronectin receptors (19). Finally, Russell *et al.* (20) used Southern blot analysis to show that there were no major insertions or deletions in either the GPIIb or GPIIIa genes in either the Iraqi-Jewish or Arab thrombasthenic patients and suggested that a more subtle defect was likely to be the cause of the disease in these two patient populations.

The recent discovery that platelet-specific mRNA sequences can be amplified by the PCR (21) has made it technically feasible to investigate the primary structure of platelet-specific gene products. We have previously applied this technology to identify small, naturally occurring sequence variations present in the genes for GPIIb and GPIIIa that result in the formation of the human platelet alloantigens P1^{A1}/P1^{A2} (22) and Bak^a/Bak^b (23). In this article, we report the application of platelet PCR technology to the determination of the molecular basis of Glanzmann's thrombasthenia in the Iraqi-Jewish and Arab populations living in Israel. We further report the development of a

simple, rapid PCR-based test that should prove useful in carrier detection and population screening. Our approach should be widely applicable for the examination of sequence variations associated with other clinically important inherited platelet bleeding disorders.

METHODS

Subjects. We studied 12 Iraqi-Jewish Glanzmann thrombasthenia patients from nine different kindreds and 10 Arab thrombasthenics from five different kindreds. All of the patients have been described as part of a detailed clinical, immunochemical, and biochemical analysis of Israeli thrombasthenia patients (14, 16–19, 24, 25). All of the patients have severe Glanzmann thrombasthenia with virtually no immunodetectable platelet GPIIb–IIIa complex, no platelet aggregation in response to ADP, and no clot retraction.

Amplification and Analysis of Platelet mRNA Sequences. Platelet RNA was prepared from patient and control blood by using a modified version of the technique of Chomczynski and Sacchi (26), as described (23). First-strand cDNA was synthesized from specified regions (detailed below) of GPIIb and GPIIIa using GPIIb- or GPIIIa-specific antisense primers and Moloney murine leukemia virus reverse transcriptase according to published methods (21–23). cDNA was then amplified by the PCR according to the method of Newman and colleagues (23). In several experiments, both cDNA synthesis and PCR were performed with two different primer pairs in the same tube (duplex PCR), with one primer pair corresponding to GPIIb and the other corresponding to GPIIIa. This allowed assessment of relative abundance of mRNAs encoding these two glycoproteins in control and patient samples. After electrophoretic separation and analysis of PCR products on agarose gels, the appropriate bands were excised and recovered with GeneClean (Bio 101, La Jolla, CA). DNA was subjected to direct sequence analysis with T7 DNA polymerase (United States Biochemical) and standard dideoxynucleotide double-stranded sequencing techniques according to the manufacturer's directions. Occasionally, sequence analysis was performed on gel-purified PCR products that had been subcloned into the plasmid vector pGEM 5Zf (Promega Biotech).

Genotypic Analysis of Control and Patient Genomic DNA Samples. High molecular weight genomic DNA was extracted from whole blood by the method of Miller *et al.* (27). For population screening and carrier detection, oligonucleotide primers were designed to flank the regions found to be defective in the Arab and Iraqi-Jewish thrombasthenic genes; these were then used for PCR amplification of genomic DNA from other thrombasthenic individuals within these two populations. Mutations were detected by dot-blot hybridization analysis of the PCR products with the oligonucleotide probes detailed in Figs. 2B and 3B.

RESULTS

Platelets from Both Arab and Iraqi-Jewish Thrombasthenic Patients Contain Apparently Normal Levels of GPIIb and GPIIIa mRNA. Previous studies of the thrombasthenic defect in Arab and Iraqi-Jewish patients showed that although their genes encoding both GPIIb and GPIIIa are free of detectable insertions or deletions by Southern blot analysis (20), the GPIIb–IIIa complex is not expressed on the platelet surface (17). To assess whether the lack of expression was caused by a defect in transcription of DNA into mRNA, we prepared platelet RNA from Iraqi-Jewish and Arab thrombasthenic patients and subjected it to duplex PCR analysis. Two different amplifications, one of GPIIb mRNA (from bases 4–487) and one of GPIIIa mRNA (from bases 1666–2237), were performed in the same tube on each platelet RNA

sample. In this manner, an internal control was built into each sample analyzed, and relative levels of GPIIb and GPIIIa mRNAs could be assessed simultaneously. As shown in Fig. 1, platelet RNA derived from both a normal control (lane B) and an Arab thrombasthenic individual (lane A) contained approximately the same levels of both GPIIb and GPIIIa mRNA transcripts. Similar results (data not shown) were obtained by using platelet RNA derived from an Iraqi-Jewish thrombasthenic patient. Moreover, oligonucleotide primer pairs specific for both 5' and 3' regions of these mRNA molecules were equally effective in amplifying GPIIb and GPIIIa transcripts from patient samples, suggesting that the mRNAs were intact, full-length species. Thus, transcription of GPIIb and GPIIIa messenger RNAs appears to be normal in both patient groups.

The Molecular Basis of Glanzmann Thrombasthenia in Israeli Arabs. Whereas our previous studies showed that the platelets from Arab patients have normal or increased vitronectin receptor ($\alpha_v\beta_3$) expression (19), this suggested that the GPIIIa subunit was normal. Since transfection studies have shown that a defect in either GPIIb or GPIIIa can result in deficient surface expression of the GPIIb–IIIa complex (12), we reasoned that a subtle mutation in the gene encoding GPIIb was most likely responsible for Glanzmann thrombasthenia in the Arab population. Analysis of the amplified GPIIb cDNA sequence from one patient (Fig. 2A) revealed an 18-base deletion in GPIIb mRNA from nucleotides 410–427 that results in an in-frame deletion of amino acids 106–111. Inspection of this region of the GPIIb gene (28) revealed that the missing nucleotides formed the 5' boundary of exon 4. Since an AG dinucleotide preceded the start of Arab exon 4, it suggested that deletion or alteration of the normal AG splice acceptor site immediately upstream from the beginning of the normal, intact exon 4 might be the actual cause of the defect. Thus, two primers were designed: the forward primer encompassing the 5' boundary of exon 3 (5'-TCAGCGC-CCCACCCCTTCTT-3') and the reverse primer (5'-AAAGC-AGCTACCTACGGGCGTC-3') within exon 4, in order to amplify this entire region from genomic DNA. As shown in Fig. 2A, when this region was subjected to DNA sequence analysis, a 13-base deletion encompassing the AG splice acceptor site for exon 4 was discovered. Thus, deletion of the normal AG splice acceptor site leads to the use of an alternative, normally cryptic, AG splice acceptor 16 nucleotides downstream, as schematically depicted at the top of Fig. 2. The result of this abnormal splicing event is a GPIIb mRNA transcript having an 18-bp deletion, as experimentally shown. The resulting protein product lacks 6 amino acids, including a single cysteine residue that is likely to be important in the normal folding and subsequent intracellular processing of the GPIIb–IIIa complex.

FIG. 1. PCR analysis of platelet GPIIb and GPIIIa mRNA. Total cellular RNA was prepared from platelets isolated from a control (lane B) or an Arab thrombasthenic patient (lane A). Duplex PCR analysis was performed to assess the relative abundance of GPIIb versus GPIIIa mRNA transcripts. Platelets from both the Arab patient shown as well as an Iraqi-Jewish thrombasthenic individual (not shown) contain apparently normal levels of each mRNA species.

FIG. 2. Analysis of the GPIIb mRNA and gene in Arab Glanzmann thrombasthenia (GT). (A) Nucleotide sequence analysis of amplified platelet GPIIb RNA (Right) revealed an 18-base deletion from nucleotides 410–427, resulting in the deletion of amino acids 106–111 (ACAPWQ). Analysis of the genomic DNA sequence in this region (Left) showed 13 nucleotides missing at the 5' boundary of exon 4 of the GPIIb gene, resulting in abnormal splicing to a normally cryptic AG splice acceptor within exon 4. bp, Base pair. (B) Dot-blot hybridization analysis of PCR-amplified genomic GPIIb DNA encompassing the affected region, using digoxigenin-labeled oligonucleotide probes corresponding to both normal and mutant sequences. Data from three unrelated Arab patients and five normal kindreds are shown.

To examine the genotype of other affected Arab thrombasthenic patients, oligonucleotide primers specific for the normal or mutant sequence were designed and used to probe by dot-blot hybridization analysis the PCR products of amplified genomic DNA samples derived from five normal and nine Arab thrombasthenic patients. A representative dot-blot hybridization analysis is shown in Fig. 2B. Using this technique, we found that the deletion described above is present in the family members of three of five Arab kindreds that took part in this study. The nature of the defect(s) in the other two Arab families is not yet known.

The Molecular Basis of Glanzmann Thrombasthenia in Iraqi Jews. The platelets from Iraqi-Jewish thrombasthenic patients contain small amounts of proGPIIb but no detectable GPIIIa (14), and their platelets are deficient in vitronectin receptors as well as GPIIb-IIIa (19). These data suggest that the molecular defect is most likely within the GPIIIa gene in this population. Amplification and sequence analysis of the GPIIIa mRNA transcript from one Iraqi-Jewish thrombasthenic individual revealed an 11-base deletion within exon 12 of the GPIIIa gene (29). As shown in Fig. 3A, this mutation

results in a shift in the reading frame that leads to the synthesis of two abnormal amino acids, followed by premature termination of the GPIIIa protein shortly before the putative transmembrane domain. We also designed a dot-blot screening assay for genomic DNA from the Iraqi-Jewish patients by using a forward primer within intron 11 (5'-CACAAACGGGTAGGTAGTGAAGCC-3') and a reverse primer in intron 12 (5'-CCTAACATGGTTCTCCCTCTCC-3') such that all of exon 12, including the splice donor and splice acceptor sites, could be amplified and characterized. Analysis of an additional 11 affected Iraqi-Jewish individuals from 6 kindreds by dot-blot hybridization analysis (Fig. 3B) showed that all of the Iraqi-Jewish thrombasthenics had the same defect. Moreover, since this defect is in the patients' genomic DNA, they presumably cannot synthesize normal vitronectin receptors in any other tissues.

DISCUSSION

The purpose of the present investigation was to discover the molecular basis of Glanzmann thrombasthenia in the Iraqi-

FIG. 3. Analysis of the GPIIIa platelet mRNA and gene in Iraqi-Jewish Glanzmann thrombasthenia (I-J GT). (A) Nucleotide sequence analysis of amplified platelet GPIIIa RNA revealed an 11-base deletion starting at base 2051. This deletion produces frameshift leading to protein termination shortly before the transmembrane domain. CR, cysteine-rich region; TM, transmembrane domain. (B) Dot-blot hybridization analysis of PCR-amplified genomic GPIIIa DNA encompassing the affected region, using probes corresponding to both normal and mutant sequences, permitted identification of the observed defect in all affected Iraqi-Jewish kindreds examined. The results from seven patients belonging to six different unrelated kindreds are shown.

Jewish and Arab populations living in Israel. Based on the application of platelet RNA duplex PCR technology, we first demonstrated that the platelets from affected individuals in both of these two populations contain apparently normal proportions of amplifiable GPIIb and GPIIIa mRNA molecules. This observation made it very unlikely that the defect(s) in these patients was related to either abnormal transcription or mRNA stability. Subsequent DNA sequencing of selected regions allowed us to identify the abnormalities.

Among the Arab thrombasthenics, we found a 13-base deletion in the gene for GPIIb at the beginning of exon 4 that results in a forced alternative splicing event. As a consequence, six amino acids, including Cys-107, are deleted from the mature GPIIb protein. Since the deletion is in-frame, the remainder of GPIIb could theoretically be synthesized, complex with GPIIIa, and be expressed on the platelet surface. No platelet surface GPIIb-IIIa complex can be identified, however, suggesting that Cys-107 is critical to the normal folding of GPIIb and its ability to complex with GPIIIa. The small amounts of residual platelet pro-GPIIb that can be detected by sensitive immunoblot analysis in these patients (18) is consistent with our molecular biologic data, indicating that the mutated protein is synthesized but fails to undergo the posttranslational cleavage of GPIIb into

heavy and light chains. It is likely that complex formation with GPIIIa and subsequent transport to the Golgi apparatus are impaired (9-12).

Iraqi-Jewish thrombasthenia is due to an 11-base-pair deletion in the gene for GPIIIa. Unlike the Arab defect, this deletion occurs within the middle of an exon and results in a change in the reading frame of the GPIIIa mRNA transcript. As a consequence, GPIIIa protein synthesis is terminated shortly before formation of the putative transmembrane domain, which is thought to anchor the glycoprotein within the plasma membrane. Since no GPIIIa is detectable either on or within these patients' platelets (14), this altered form of GPIIIa is either extremely unstable and rapidly degraded after its biosynthesis, or it is secreted from the megakaryocyte as a soluble protein. Thus far, we have been unable to identify a soluble, truncated form of GPIIIa in the plasma of these patients. The impact of this mutation on biosynthesis of GPIIIa and GPIIb-IIIa complex formation may also be testable by reconstructing the Iraqi-Jewish mutation in a heterologous cell culture expression system in which the culture medium could be analyzed for the presence of the truncated form of GPIIIa.

The inability of these patients to synthesize normal GPIIIa implies that they must also be deficient in the vitronectin

receptor ($\alpha_v\beta_3$). Since the patients' only abnormality seems to be in platelet function, this raises the issue of the function of the vitronectin receptor in other tissues. It is possible that other receptors can substitute for the $\alpha_v\beta_3$ in the other tissues, especially since an "alternative" vitronectin receptor ($\alpha_v\beta_3$) has been identified (30, 31). Alternatively, the $\alpha_v\beta_3$ receptor may be uniquely responsible for some functions, but the Iraqi-Jewish patients have a compensatory alteration that diminishes the expression of the abnormalities. Other patients with Glanzmann thrombasthenia due to GPIIIa defects have recently been reported (32, 33) and studies of this group of patients should offer important insights into these alternative explanations.

Based on the specific deletions found to give rise to thrombasthenia in the Arab and Iraqi-Jewish populations, we devised a dot-blot hybridization test for these forms of Glanzmann thrombasthenia by using genomic DNA. The potential utility of this method for both pre- and postnatal testing was demonstrated by our ability to analyze the GPIIb and GPIIIa genotype of 22 thrombasthenic individuals from 14 different kindreds. The use of these oligonucleotide probes should facilitate screening of a large number of potentially affected individuals, including carrier detection in couples contemplating having children, and prenatal diagnosis for women at risk of bearing affected children. In addition, identification of the mutations affecting these two large, well-described thrombasthenic populations living in Israel provides a unique opportunity to examine the role of the integrin GPIIb-IIIa complex in mediating the potential contribution of platelets to cardiovascular disease. Using the molecular probes and the amplification strategy developed above, it should now be possible to screen these two populations and determine whether carriers of Glanzmann disease, whose platelets contain only $\approx 60\%$ of the normal number of GPIIb-IIIa receptors (17), are protected from occlusive cardiovascular disease. Study of the effects of decreased integrin function on platelet pathophysiology in these two populations may thus provide important insights into the potential utility of anti-platelet agents that can block the GPIIb-IIIa receptor on a chronic basis in preventing morbidity and mortality.

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