

Polymorphism of the glycoprotein Ia and IIIa in the group of women in childbirth does not correlate with an increased risk of developing thrombosis

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Abstract

OBJECTIVE: The aim of the study was to evaluate the prevalence of the GP Ia and GP IIIa polymorphisms in the group of women in labor, and to assess the risk of thrombosis associated with their occurrence.

DESIGN AND SETTINGS: 245 women in labor hospitalized between 1.01.2001 and 31.12.2003 r. were enrolled in the study. Patients were qualified for the study if detailed physical exam and past medical history excluded existence of known risk factors predisposing to thrombosis. Study group was composed of 72 women in childbirth, which at some point during current pregnancy or in early labor were diagnosed with thrombosis, and control group included 173 women in labor randomly picked from the group of patients with uncomplicated pregnancies. Polymorphic regions of platelets glycoprotein were detected using genotyping methods based on polymerase chain reactions (PCR).

RESULTS: 1,72% of patients were found to have thrombosis. The thrombosis was located in the venous system in 97,2% of cases. Arteries were affected in two patients (2,7%). Prevalence of individual platelets glycoprotein mutations did not differ between controls and study group. In both groups platelets glycoprotein polymorphisms moderately pro-thrombotic A1/A2 and C/T dominated, and the least numerous were strongly pro-thrombotic A2/A2 and T/T.

CONCLUSIONS: Our results did not show the causative relationship between the existence of platelets GP IIIa / GP Ia mutations and venous system thrombosis in the women in labor. Probably presence of pro-thrombotic mutations of platelets glycoprotein in this group of patients does not represent the increased risk of thrombosis.

Abbreviations:

GP – glycoprotein
 OD – optical density
 PCR – polymerase chain reaction
 DNA – deoxyribonucleic acid

Introduction

Platelets response to the initiation of the clotting process is multistage, and results in formation of a haemostatic plug. Platelets adhesion to the endothelium of damaged blood vessels, their spreading, secretion as well as the redistribution of plasma membrane phospholipids resulting in phosphatidylserin exposure, represents their different functions. [2, 7]. Formation of the platelet plug is mediated through glycoprotein receptors. Two of them glycoprotein Ia (GP Ia) and glycoprotein IIIa (GP IIIa) represent the integrins, which polymorphism by some investigator was reported to be an inherited risk factor for acute coronary events [18].

Glycoprotein Ia mainly appears in the Ia-IIa complex and enables formation of the cytoskeleton of the clot. Several GP Ia polymorphisms associated with coagulation disorders as effect of point mutations in the genome coding the glycoprotein, was described. Description of the polymorphism which determines occurrence of the platelets HPA-5 antigen system was one of the first one published [5]. This particular one is related to the change of guanine for alanine in cytoplasmic Deoxyribonucleic acid (cDNA) which corresponds to the appearance of glutamine glycoprotein (variant a) or lysine glycoprotein (variant b) in the position 505. Clinically this polymorphism may present as alloimmune thrombocytopenia in the neonate, post transfusion purpura, post transfusion immunity to the platelets, or immune mediated thrombocytopenia after bone marrow transplantation [5, 19]. In the recent years new clinically significant polymorphisms in the Ia glycoprotein coding region were discovered. These are: a change of nucleotide in position 807 C/T – in the part of the chain responsible for coding phenylalanine in position 224 of glycoprotein Ia, a change of nucleotides in the position 873 A/G – in the part of the chain responsible for coding tyrosine in the position 246 of glycoprotein Ia, and a change of nucleotides in position 837 T/C in the part of the chain responsible for coding alanine in the position 234 of glycoprotein Ia. Although above described polymorphisms do not produce changes in the amino acid sequence of the glycoprotein polypeptide chain, they do however affect the number of copies of the Ia-IIa receptor complex in the platelets and in so doing, modify their function. Very interesting conclusions can be drawn from the results of the studies based on the identification of the Ia-IIa complexes using tagged monoclonal antibodies anti - $\alpha_2\beta_1$ and then correlation of the results with the genetic tests. Assuming linkage of the genes coding integrin Ia, three haplotypes were found: Type I – (807T/837T/873A/Br^b), Type II – (807C/837T/873G/Br^b), and Type III – (807C/837C/873G/Br^a). The first type correlates with a higher level of the Ia-IIa complex, the other two with a low level of Ia-IIa. Clinically presence of the first haplotype is

associated with higher platelet adhesion potential and indirectly with intensification of prothrombotic platelet mechanisms. Above presented assumptions became the basis for the evaluation of platelets adhesion activity using genetic testing, by determining the frequency of gene polymorphisms coding glycoprotein Ia mainly 807 C/T.

The integration of heterodimer integrin composed of glycoprotein IIb and glycoprotein IIIa (GP IIb-IIIa), with fibrinogen, von Willebrand factor or fibronectin is possible only after platelet activation [2, 4, 9, 15]. Additionally, platelet activation leads to potentialization of their adhesive abilities by the mechanism of translocation of the intracellular pool of GPIIb-IIIa to the cell surface. It has been shown that presence of the arginine-glycine-asparagine acid sequence (RGD) in the ligand molecule is required for binding with GP IIb-IIIa complex. RGD sequence was discovered in all ligands for GP IIb-IIIa so far. Moreover, it's been proven, that other proteins, which molecule contains that sequence are also able to bind to a GP IIb-IIIa complex. These proteins, which belong to the disintegrin family, on binding with GP IIb-IIIa complex, deactivate it, and thereby making the platelets incapable of adhesion [14]. The P1^A antigen system represented by two alleles P1^{A1} and P1^{A2} deserves particular attention among other GP IIb-IIIa polymorphisms. Allele P1^{A1} coding for leucine in position 33 is more prevalent in the population. Allele P1^{A2} is responsible for the presence of proline in the same position. The relationship between the occurrence of cardiac complications and presence of allele P1^{A1/A2} PI of GP IIIa has been suggested. However, based on available data, especially lack of prospective studies, one cannot conclude that this polymorphism constitutes a hereditary risk factor for thrombosis. Up to now three genotypes of GP IIIa are described: two homozygous A1/A1 and A2/A2, and heterozygous A1/A2 [9, 18].

Materials and method

Study patients and control. 245 women hospitalized in the Fetal-Maternal Medicine and Gynecology Clinic of the Polish Mother's Health Research Institute in Lodz from 1.01.2001 to 31.12.2003 r. was enrolled in the study. For the same time period there were 4162 deliveries in our institution. Women in labor were qualified for the study if detailed physical exam and past medical history excluded existence of known risk factors predisposing to thrombosis. The following risk factors were taken into consideration: cigarette smoking, obesity, metabolic diseases, especially diabetes, and infections. Furthermore, women in childbirth with past medical history positive for thrombosis were excluded from the control group. Study group was composed of 72 women in childbirth, which at some point during current pregnancy or in early labor were diagnosed with thrombosis. Diagnosis was based on the presence of clinical signs, and confirmed by the Doppler studies showing thrombus formation in artery or vein. Control group included 173 women in labor randomly

picked from the group of patients with uncomplicated pregnancies. In that group of patients initial data gathered from the history, and physical exam excluded the existence of thrombosis or hereditary pro-thrombotic polymorphisms.

Extraction of the DNA. DNA was isolated from a whole blood sample collected into EDTA K₂ tube, and equal volume of buffer was added for lyses (0.32 saccharose, 10 mM TRIS, 2 mM MgCl₂, 1% Triton X-100, pH=7.5). After intense mixing, sample was centrifuged at 11000 × g for 1 min. at 4°C. Supernatant was discarded, and sediment was rinsed many times using the buffer for lyses, until it became white. Then the buffer composed of 50 mM KCl, 10 mM TRIS, 1 mM MgCl₂, 0.01 mg/ml gelatine, 0.45% Tween 20, pH 8.3, was poured over the sediment, proteinase K was added to the final concentration of 100 µg/ml, and mixture was incubated first for 1 hour at a temperature of 56°C, then for 10 minutes at a temperature of 95°C. Afterwards mixture was centrifuged at 11000 × g, for 1 minute, supernatant was collected (DNA solution) and stored at a temperature of -20°C. The DNA concentration was estimated with spectrophotometric method based on the relationship that 1 unit of optical density (OD) corresponds to 50 µg/ml of double stranded DNA.

Detection of glycoprotein polymorphism. Polymorphic regions of platelet glycoproteins were detected using genotyping methods based on polymerase chain reactions (PCR). Products of PCR was subjected to restrictive enzymatic digestion (RFLP-PCR method). Products of digestion are identified by unipolar agarose electrophoresis, and stain with ethidine bromide. For GP Ia polymorphisms detection, 115 bp fragment of the gene for the GP Ia subunit of the collagen receptor containing polymorphism 807 C/T was amplified by the PCR method using the following oligonucleotide starters: 5'-GTG TTT AAC TTG AAC ACA TAT -3' and 5'-ACC TTG CAT ATT GAA TTG CTT-3' in the thermocycler T3 (Biometra, Germany). DNA amplification reaction consisted of several stages: so called hot start with initial denaturation (85°C, 4 min), 30 cycles of amplification which comprised denaturation (95°C, 30 s), incorporation (50°C, 30 s), elongation (72°C, 60 s). Reaction mixture (25 µl) contained: 1 ng genomic DNA, 200 µM of each dNPT, 5 pmol of each starter and 1 unit *Taq* of DNA polymerase in 4 mM MgCl₂, 50 µM KCl, 10 µM Tris-HCl, pH 8.3. Determination of the glycoprotein GP IIIa polymorphism was based on the method described by Weiss E.J et al. [18]. Fragment 106 p.z. of the exon 2, of the gene coding GPIIIa was amplified by the PCR using a starter oligonucleotide sequences: 5'-TGGACTTCTCTTTGGGCTCCTGACTTAC-3', and 5'-CGATGGATTCTGGGGCACAGTTATC-3' in the thermocycler T3 (Biometra, Germany). Reactive mixture (25 µl) contained: 1 µg genomic DNA, 200 µM each of the dNTP, 50 pmol of each starter, 1.5 µM MgCl₂, 50 µM KCl, 10 µM Tris-HCl, pH=8,3, 1 Unit *Taq* DNA polymerase. Thermal profile of the PCR reaction included: initial denaturation (96°C, 4 min); then 35 cycles, each composed of DNA denaturation (94°C, 30 s), incorporation of the starters (58°C, 30 s),

elongation (72°C, 30 s); and final elongation (72°C, 7 min).

Digestion and detection of the restrictive fragments. PCR product (12 µl) was digested with the restrictive enzyme *Msp I* (2 units) in the appropriate buffer (33 mM Tris-acetic acid; 10 mM magnesium acetate; 66 mM potassium acetate; 0,1 mg/ml bovine albumin; pH=7,9, 37°C) for 18 hours in the temp. 37°C. Electrophoresis of the restrictive fragments was conducted on 12% polyacrylamide gel (acrylamide: bisacrylamide 9,5: 0,5 weight ratio) prepared in the TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM disodium versenate). TBE was also making electrophoretic buffer. Electrophoresis was conducted in the Mini-Protean II system (Bio-Rad, Germany) with a separation pathway 7 cm, voltage 12 V/cm (initially), and 15 V/cm (after sample entering gel) for 1 hour. Gel was stained with ethidine and examined in the UV light. Parallel to the electrophoresis separation of the standard for the molecular weights was conducted. Documentation and gel's analysis was done using *Gel Doc 2000* system (Bio-Rad, Germany).

PCR product (10 µl) was digested with restrictive enzyme *Taq I* (3 units.) for 2 hours, at 65°C in the buffer composed of: 10 mM Tris-HCl (pH 8), 5 mM MgCl₂, 100 mM NaCl, and 0.1 mg/ml BSA. Electrophoresis of the restrictive fragments was conducted on 12% polyacrylamide gel (acrylamide: bisacrylamide 9,5: 0,5 weight ratio) prepared in the TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM disodium versenate). Electrophoresis was conducted in the Mini-Protean II system (Bio-rad, Germany) with a separation pathway 7 cm, voltage 12 V/cm (initially), and 15 V/cm (after sample entering gel) for 1 hour. Gel was stained with ethidine. Documentation and gel's analysis was done using *Gel Doc 2000* system (Bio-Rad, Germany).

Statistical analysis. Statistical analysis was based on the evaluation of the rate of appearance of the studied polymorphisms of the platelets glycoproteins in the study group and controls. Statistical significance of observed differences was evaluated using Chi-square analysis, Fisher test, or Mann-Whitney-Wilcoxon test. The difference was statistically significant if p value was < 0,05.

Results

In analyzed population 1, 72% of women in labor were found to have thrombosis. In 97,2% of cases thrombosis was located in the venous system, and in all cases thrombi were found only in the left lower extremity. Arterial thrombosis was diagnosed in two cases (2,7%), and in both patients arteries of the upper extremities were involved. Age of the patients in the control group was (mean, ± SD) 28,3, ± 4,9 years. Patients in the study group were older, their age was (mean, ± SD) 30,4, ± 5,3 years. The difference was statistically significant. (p=0,004) (Table 1). In the control group women delivered at 38,3, ± 2,4 (mean, ± SD) weeks. In the study group pregnancy lasted 37,9 ± 2,7 (mean, ± SD) weeks. Difference was not statisti-

Table 1. Clinical characteristics of the patients

Parameters	Controls		Study group		Test results
	Mean	SD	Mean	SD	
Age	28,3	4,9	30,4	5,3	Mann-Whitney-Wilcoxon test p=0,004
Weeks of pregnancy at delivery	38,3	2,4	37,9	2,7	Mann-Whitney-Wilcoxon test p=0,325

Table 2. Clinical characteristics of the patients

Parameters	Controls		Study group		Test results
	N	%	N	%	
Parity					
Primiparas	76	43,9	11	15,5	Fisher test p<0,001
Multiparas	97	56,1	61	84,5	
Mode of delivery					
Normal spontaneous vaginal delivery	157	90,8	41	56,9	Fisher test p<0,001
Cesarean Section	16	9,2	31	43,1	

cally significant. (p=0,325) (Table 1). Percentage of primiparas in the control group (43,9%) was higher than in the study group (15,5%) and this difference was statistically significant (p<0,001). Mode of delivery was also evaluated. In the control group 90,8% of women delivered via normal spontaneous vaginal delivery, while only in 56,9% of patients in the study group pregnancy ended with this mode of delivery. Difference was statistically significant (p<0,001). However, thrombosis was not considered an indication for the cesarean section. Among platelets glycoprotein polymorphisms moderately pro-thrombotic heterozygous A1/A2 and heterozygous C/T dominated. The least numerous were strongly pro-thrombotic homozygous A2/A2 and T/T. Table 3 presents different genotypes of platelets GP Ia and GP IIIa glycoprotein found in our patients. It was very interesting that percentage of individual genotypes in the control and study group was almost identical. Statistical analysis and comparison between groups was done separately for all three genotypes of GP Ia and GP IIIa. No statistically significant difference was found. Prevalence of homozygous alleles A1/A1 and CC, as well as pro-thrombotic alleles A1/A2, A2/A2, and C/T, T/T in controls and study group was also compared. Again the difference was not statistically different. In both groups most prevalent was homozygous variant A1/A1 and heterozygous C/T. Prevalence of individual platelets glycoprotein mutations did not differ between controls and study group, nevertheless parameters defining the risk of thrombosis in women in labor with pro-thrombotic platelets glycoprotein GP IIIa and GP Ia mutations were evaluated. Results did not confirm the causative relationship between the existence of platelets glycoprotein GP IIIa / GP Ia mutations and venous system thrombosis in the women in labor. Presence of pro-thrombotic mutations of platelets glycoprotein in women in labor, probably does not represent the increased risk of thrombosis in these group of patients.

Discussion

Thrombo-embolic disease in the women in labor is one of the very important complications occurring during the course of the labor and delivery [13, 14]. According to statistic data from many countries it is the number one cause of death related to pregnancy [2, 10, 14, 17]. Estimated overall prevalence of the thrombo-embolic complications in the women in labor is 0,1%–0,2%. In the group of women which delivered via Cesarean Section these complications were diagnosed in 1,8% of patients [10].

High rate of thrombo-embolic complications found in our population of patients (1,72%), much different from the data published by other authors, can be explained by the profile of the patients hospitalized in the Polish Mother's Health Research Institute in Lodz. Institute serves as a referral center for high risk pregnancies from the macro region. It is well known, that the risk of thrombo-embolic complications especially in the venous system increases 5-fold during pregnancy [10, 14].

Pro-thrombotic factors associated with pregnancy include physiological changes of homeostasis, venous stasis in the lower extremities related to the increased intra abdominal pressure, decreased vessel walls tension secondary to some hormonal changes associated with pregnancy, perinatal or surgical trauma, and in some cases thrombophilia. [3, 10, 14, 17, 20]. According to some authors, thrombo-embolic complications in the venous system in the women in labor with thrombophilia are associated with the deficiency of one, or several natural coagulation inhibitors (antithrombin III, protein C, protein S), Leiden V factor mutation, G20210A prothrombin gene mutation [1, 3, 8, 19, 20].

Our observations are in agreement with previous study showing that thrombi in the venous system are most commonly found in the vessels of the left lower extremity (femoral/iliac vein) [14]. In our material

Table 3. Summary of studied GP IIIa and GP Ia glycoprotein polymorphisms

Paramter	Controls		Study group		Test results
	N	%	N	%	
GP IIIa					
A1/A1	130	76,5	53	76,8	Fisher test p=1,00
A1/A2	38	22,4	15	21,7	
A2/A2	2	1,2	1	1,4	
A1/A1	130	76,5	53	76,8	Fisher test p=1,00
A1/A2 and A2/A2	40	23,6	16	23,1	
GP Ia					
CC	61	35,7	24	34,3	Fisher test p=0,935
CT	93	54,4	38	54,3	
TT	17	9,9	8	11,4	
CC	61	35,7	24	34,3	Fisher test p=0,935
CT and TT	110	64,3	46	65,7	

Table 4. List of parameters for the risk of thrombosis in the women in child birth with platelets glycoprotein polymorphism.

	Polymorphism GP Ia	Polymorphism GP IIIa
Odds ratio (OR)	1,062	1,014
Relative risk (RR)	1,021	1,017
Assigned (AR)	0,010	0,002
Proportional assigned risk (PAR)	0,020	0,010
Relative risk (χ^2)	0,019	0,002

veins of the left lower extremities were most commonly involved, and only in two cases thrombo-embolic process developed in the arterial vessels. Slow blood flow in the venous system, secondary to hemodynamic changes caused by enlarging uterus are predisposing pregnant women to the thrombosis.

In our study percentage of pro-thrombotic platelets glycoprotein mutations in the study and control group were not statistically different, and prevalence of individual genotypes was comparable with the data published by other investigators [11]. Thrombo-embolic complications in the arterial system are very rarely observed in the pregnant women [10], and usually develop in the people who are carriers for the pro-thrombotic platelets glycoprotein GP IIIa and GP IIa mutation [5, 6, 11, 15, 16, 18].

Lanska D.J.et al. reviewed the national data from 50 million deliveries in USA. They've found 17,7 cases of stroke, and 11,4 cases of intracranial venous thrombosis per 100 000 deliveries [12]. In that study, pregnant women who developed thrombo-embolic complications, all had additional risk factors like antiphospholipid syndrome, ovaries hyper stimulation syndrome (in vitro fertilization), pregnancy associated hypertension, preeclampsia. In our material thrombo-embolic complications in the arterial system occurred in 0,05% of cases, and developed in both cases in the arterial vessels of the upper extremities. When compared to the data published in the literature, prevalence rate in our study was lower. One possible explanation might be related to the fact that pregnant women qualified for our studies did not have any other known risk factors for the

thrombo-embolic complications. Studies performed in the number of centers indicate that pro-thrombotic platelets glycoprotein GP IIIa and GP IIa mutations are one of the risk factors for the coronary artery disease and stroke [6, 11, 15, 18, 21]. We did not observe these complications in our patient, even though the prevalence of individual mutations in our study was similar to reported by others [6, 11, 15, 16, 18, 21]. It is possible that different percentage of thrombo-embolic complications observed in the group of patients we analyzed is related to their lower mean age. This thesis as well as a possibility of existence of other protective mechanisms associated with pregnancy and puerperium decreasing the frequency of this type of complications requires further studies. Because of a small number of patients in our study more meaningful statistical analysis could not be done. At this time it is difficult to unequivocally determine the role of GP Ia and GP IIIa polymorphisms in the pathogenesis of thrombo-embolic complications. Many investigators believe that presence of the pro-thrombotic platelets glycoprotein genotypes by itself does not increase the risk of thrombo-embolic complications. Our results are in agreement with above stated thesis. Surprisingly prevalence of individual platelets glycoprotein genotypes potentially pro-thrombotic, and not associated with thrombosis in controls and study group were similar. So, probably causative relationship between the presence of specific genotype and thrombosis does not exist. Pathogenesis of thrombo-embolic disease in pregnant women is probably complex, and determined not only by the presence of pro-thrombotic polymorphisms of the platelets glycoprotein. Thrombo-

embolic complications in our group of women in labor, manifested mainly in the venous system, are probably related to other factors than polymorphisms of the GP Ia/ GP IIIa.

In conclusion, prevalence of pro-thrombotic polymorphisms of platelets glycoprotein GP IIIa /GP Ia is similar in the group of women in labor with thrombosis and without thrombosis. Polymorphisms of platelets glycoprotein GP Ia and GP IIIa are not the genetic risk factor for developing thrombosis in the women in labor.

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