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LABORATORY STUDY

The platelet glycoprotein Ia/IIa gene polymorphism C807T/G873A: a novel risk factor for retinal vein occlusion

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Abstract

Retinal vein occlusion (RVO) is associated with hyperhomocysteinaemia and the antiphospholipid syndrome—disorders known to contribute to both arterial and venous thrombosis. In both of these conditions and RVO, platelet activation occurs. Aspirin, not warfarin, is the most effective antithrombotic agent in RVO and, taken together, these observations suggest an important role for platelets in this common ocular thrombotic condition. Platelet glycoprotein Ia/IIa (GpIa/IIa) is an adhesion molecule mediating platelet–collagen interactions and is key to the initiation of thrombosis. Recently, the cellular density of this molecule was shown to be determined by two silent, linked polymorphisms (C807T/G873A) within the GpIa/IIa gene. There is evidence that some of the resulting genotypes are associated with thrombo-embolic disease. This study therefore aimed to establish the prevalence of the GpIa/IIa polymorphisms and the three commonest hereditary thrombophilic disorders (prothrombin gene G20210A (PT) mutation, Factor V Leiden (FVL), and the thermolabile methylene tetrahydrofolate reductase C677T (MTHFR) mutation) in patients with RVO and normal controls. The GpIa/IIa polymorphisms and thrombophilic abnormalities were all identified using the polymerase chain reaction. Our results show that the frequency of the GpIa/IIa polymorphisms was similar in our normal control population to previously published series. Patients with RVO, however, had only a 10% (4/40) frequency of the lowest risk subtype (CC/GG) compared to 37.5% (15/40) in the control group—P 0.0039. The incidence of the PT, FVL, and MTHFR thrombophilic mutations was not different between the two groups, but interestingly none of the 7/40 RVO cases with a PT, FVL, or MTHFR mutation had the low-risk GpIa/IIa genotype while all but one of the controls did—$P<0.05$. Thus, 17.5% of RVO patients harboured more than one prothrombotic abnormality. The principal difference between the RVO and control group was the very high incidence of the intermediate-risk GpIa/IIa subtype (CT/GA)—82.5 *vs* 50%, $P<0.05$. These results suggest a major role for GpIa/IIa polymorphisms in the pathogenesis of RVO.
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Keywords: retinal vein occlusion; platelet glycoprotein polymorphisms; prothrombin gene mutation; factor V Leiden; thermolabile methylene tetrahydrofolate reductase mutation

Introduction

Retinal vein occlusion (RVO) is the commonest retinal vascular disorder presenting to ophthalmology services. The pathogenesis is multifactorial with open-angle glaucoma, retinal artery disease, and systemic illness; for example, diabetes, hypertension, hyperviscosity, arteriosclerosis, and hyperlipidaemia, all risk factors.[1–6] A role for these disorders is in keeping with Virchow's triad of haemostasis and endothelial damage. With the recent identification of common thrombophilic disorders, there has been renewed interest in the third of Virchow's triad, notably hypercoaguability. Studies looking at both rarer causes of thrombophilia (protein C,

protein S, and antithrombin III deficiency) and the three more common Factor V Leiden (FVL), prothrombin gene G20210A (PT), and homozygosity for C677T methylene tetrahydrofolate reductase enzyme mutation (MTHFR) have given conflicting results with regard to their exact role(s) in RVO.[7] Homozygous MTHFR C677T mutations can cause mildly elevated homocysteine levels, but this effect is neutralised if there is a high folate content in the diet.[8] Thus, many patients homozygous for this abnormality have normal homocysteine levels. In contrast, most studies directly measuring homocysteine find high levels to be associated with RVO.[9] In addition, another acquired thrombophilic condition, antiphospholipid syndrome, has also been identified as a risk factor for RVO. Interestingly therefore, the major thrombophilic risk factors for RVO—hyperhomocysteinaemia and antiphospholipid syndrome—cause both arterial and venous thrombosis whereas the hereditary thrombophilic risk factors usually only cause venous thrombosis.[7] While there are consistent clinical risk factors underlying RVO, to date there has been no abnormality identified in the majority of patients, that is, suggesting causality, and no report of any possible genetic marker.

Previous studies have suggested that platelet activation and antiplatelet therapy may play a role in RVO.[10–12] Both hyperhomocysteinaemia and antiphospholipid syndrome can cause platelet activation as well as thrombosis.[13,14] The platelet glycoprotein Ia/IIa (GpIa/IIa) is a membrane complex that mediates platelet adhesion to subendothelial type I and type III collagen.[15] Two silent GpIa/IIa polymorphisms 807 C→T and 873 G→A have recently been identified.[16] It has subsequently been shown that the density of the GpIa/IIa receptor depends on the particular polymorphism present with the CC/GG subtype the lowest, the TT/AA subtype the highest, and the CT/GA subtype having intermediate receptor levels.[16,17] Thus the CC/GG subtype has the lowest platelet adhesion to collagen—and hence thrombogenic potential—and the TT/AA subtype the highest. Given the importance of GpIa/IIa in primary haemostasis, several studies have assessed the roles of these polymorphisms in both large vessel venous and arterial thrombosis including myocardial infarction (MI), stroke, deep venous thrombosis, and pulmonary embolism.[18–23] Although results are conflicting, it appears that GpIa/IIa polymorphisms may play a role in large vessel arterial but not venous thrombosis and its role in microvascular disease is unknown. We know however that GpIa/IIa polymorphisms play a major role in retinal vessel disease. In a large cohort study, Matsubara *et al*[24] showed that diabetic patients with the GpIa/IIa TT/AA subtype had a 3.4-fold increase risk of developing retinopathy compared to patients with the CC/GG subtype. Patients with CT/GA had an intermediate risk.

In the present study, we re-evaluated the prevalence and potential pathogenic role(s) of the three commonest hereditary thrombophilic conditions, FVL, prothrombin gene mutation, and the MTHFR mutation, along with GpIa/IIa polymorphisms in RVO and large vessel venous thrombo-embolic patients and normal controls to obtain data on any potential role(s) in microvascular disease.

Methods

Patients

A total of 40 consecutive patients presenting with RVO to the Medical Ophthalmology Clinic at Heartlands Hospital were prospectively screened for the various genetic abnormalities. All patients underwent complete ophthalmological and medical examinations by one of the authors (PMD). Clinical parameters, including body mass index and blood pressure, were recorded. The diagnosis of definite RVO was confirmed by dilated slit-lamp biomicroscopy (PMD). Routine haematological and biochemical tests were undertaken including renal and liver function, lipids and glucose, and full blood count and viscosity. Hypertension was defined according to WHO criteria and hyperlipidaemia was defined according to the criteria of the British Hyperlipidaemic Association.[25,26]

In sum, 40 exact age- and sex-matched normal controls (age 40–84 years, median 66, M : F 21 : 19) and patients with deep venous thrombosis (DVT, age 40–85 years, median 66, M : F 21 : 19) were also screened for the various thrombophilic conditions. All DVT patients were diagnosed using Doppler ultrasound and/or venography at Heartlands Hospital and had completed a course of anticoagulant therapy (see Table 1).

Laboratory methods

Peripheral blood was taken into EDTA-containing tubes and sent to the molecular pathology laboratory at Heartlands Hospital where all molecular testing was undertaken.

DNA was extracted using a standard salting out method. The FVL polymerase chain reaction (PCR) was performed according to the method of Gandrille *et al*.[27] In brief, two primer sequences were used:

FVH 1—TCAGGCAGGAACAACACCT and FVH2—GGTTACTTCAAGGACAAAATACCTG TAAAGCT.

A 3 h restriction enzyme digest using *Hind*III was undertaken prior to electrophoresis on a 3% agarose gel.

Table 1 RVO patient characteristics

Male/female	21/19
Age (years)	Mean 66.1 (range 40–85)
CRVO/BRVO	22/18
Single/recurrent RVO	34/6
Smoker/non-smoker/ex-smoker	13/23/4
Hypertensive	7 (17.5%)
Hyperlipidaemia	15 (37.5%)
Diabetes	2 (5%)

PCR controls consisted of known GG, GA, and AA DNA samples.

The prothrombin gene 20210 mutation PCR was performed according to the method of Poort *et al*.[28] In brief, two primer sequences were used:

Pro A—TCTAGAAACAGTTGCCTGGC and

Pro B—ATAGCACTGGGAGCATTGAAGC.

A 3 h restriction enzyme digest using *Hind*III was also undertaken prior to electrophoresis on a 3% agarose gel. PCR controls consisted of known GG and GA DNA samples.

The MTHFR PCR was performed according to the method of Froost *et al*.[29] In brief, two primer sequences were used:

Hom A—TGAAGGAGAAGGTGTCTGCGGGA and

Hom B—AGGACGGTGCGGTGAGAGTG.

A 3 h restriction enzyme digest using *Hinf*1 was undertaken prior to electrophoresis on a 3% agarose gel. PCR controls consisted of known CC, CT, and TT DNA samples.

The GpIa/IIa PCR was performed similar to the method of Dinauer *et al*.[30] In brief, a two-stage multiplex PCR was performed, the first using primer sequences intron G, exon 8, 807C and 807T, and the second using intron G, exon 8, 873A and 873G.

Intron G—GATTTAACTTCCCAGCTGCCTTC.

Exon 8—CTCAGTATATTGTCATGGTTGCATTG.

807 C—GTGGGGACCTCACAAACACATGC.

807 T—ATGGTGGGGACCTCAACAAACACATAT.

873 G—GGTGGGCGACGAAGTGCTAGG.

873 A—GGTGGGCGACGAAGTGCTAGA.

Electrophoresis was carried out on a 2% agarose gel and stained with ethidium iodide. All PCR reactions were performed on a Perkin-Elmer 4800 thermal cycler. Ethical permission was obtained and all patients gave informed consent. Statistical analysis was undertaken using the χ^2 test (GF).

Results

Overall in the RVO group, we found only one patient with FVL, two with PT mutation and four homozygous for the MTHFR mutation—results not significantly different from the normal control group of patients (Table 2). The frequency of these abnormalities is, as expected, higher in the recurrent DVT group of patients compared to the normal control group, with FVL significantly higher (1 *vs* 8, $P<0.05$). All FVL and PT mutations identified were heterozygous.

Table 2 Frequency of the common hereditary thrombophilia conditions

	Normal controls	*DVT group*	*RVO group*
Factor V Leiden	1	8	1
Prothrombin gene mutation	1	3	2
Homozygous MTHFR mutation	6	5	4

In our normal population only 15/40 (37.5%) had the lowest risk CC/GG GpIa/IIa polymorphism compared with only 4/40 (10%) in the RVO group (P 0.0039, χ^2 test) and 9/40 (22.5%) of the recurrent DVT group ($P=$NS, χ^2 test, normal controls *vs* DVT group) (Table 3). Thus 90% of the RVO group had the two polymorphisms with the higher density GpIa/IIa receptor status. There was however no significant difference between the control group and RVO in terms of the highest risk polymorphism TT/AA. The difference between the two groups is because of a very high frequency of the intermediate risk GpIa/IIa status CT/GA compared to normal controls: 82.5 *vs* 50%, $P<0.001$, χ^2 test.

In the RVO group the patient with FVL, all four of the patients homozygous for the MTHFR mutation and one of the patients with the prothrombin mutation also had the intermediate risk GpIa/IIa CT/GA status. The other RVO patient with the prothrombin mutation also had the highest risk GpIa/IIa TT/AA subtype. Thus, seven of the RVO group had more than one genetic abnormality compared to only one in the normal group ($P<0.05$, χ^2 test).

Of the six patients with recurrent RVO, five had the intermediate CT/GA subtype with one also having a homozygous MTHFR mutation. The remaining patient had no apparent genetic abnormalities. There was therefore no statistical difference in the prevalence of genetic mutations between those patients with a single and those with recurrent RVO (5/6 *vs* 31/34, $P=$NS). In those patients less than 50 years of age, there was also no difference in the pattern of mutations. Similarly, there was no difference between male and female RVO patients.

Discussion

The pathogenesis of RVO is complex owing to a combination of both genetic and enviromental factors acting on the three elements of Virchow's

Table 3 Platelet GpIa/IIa genotype status

Gp Ia/IIa genotype	*Normal controls*	*DVT group*	*RVO group*
CC/GG	15 (37.5%)	9 (22.5%)	4 (10%)
CT/GA	20 (50%)	24 (60%)	33 (82.5%)
TT/AA	5 (12.5%)	7 (17.5%)	3 (7.5%)

triad—haemostasis, endothelial damage, and hypercoaguability. The commoner risk factors for RVO are similar to those seen in arterial rather than venous thrombosis, notably diabetes, hypertension, and hyperlipidaemia to which can be added local anatomical factors such as open-angle glaucoma.[1–6]

In RVO studies, the potential pathogenic role(s) of the common causes of thrombophilia, that is, FVL, prothrombin gene mutation, and MTHFR, has given conflicting results. Interestingly, the acquired thrombophilia conditions, hyperhomocysteinaemia, and antiphospholipid syndrome, which cause both arterial and venous thrombosis, are most associated with RVO.[7]

The platelet GpIa/IIa complex initiates platelet adhesion to collagen at low and high shear rates (50–1500/s), ultimately leading to thrombus formation.[31,32] Recent studies have shown that a common polymorphism with two silent linked point mutations in the GpIa/IIa complex determines the density of receptor expression and therefore platelet adhesion.[16,17] The TT/AA subtype has the highest, the CC/GG subtype the lowest, and the CT/GA subtype intermediate receptor density.

The aim of our study was to re-evaluate FVL, prothrombin gene mutation, and MTHFR and to look for the first time at platelet GpIa/IIa polymorphisms. We postulated a role for platelets from observations that aspirin, not warfarin, is the most effective anticoagulant in RVO, that platelets are activated in RVO, and that GpIa/IIa has been shown in some (but not all) studies to be a thrombotic risk factor.[10–12] In addition, the GpIa/IIa polymorphism has recently been shown to play a role in the development of retinopathy in diabetic patients.[24]

Our study confirms the earlier reports suggesting that isolated FVL, prothrombin gene mutation, and MTHFR are not major risk factors for RVO.[7] It is however the first study identifying that the GpIa/IIa genotype(s) leading to an increased receptor density is a very common abnormality in this patient group. The 37.5% incidence of the low GpIa/IIa density (CC/GG) seen in our normal population is similar (33–49%) to that reported in other normal European populations.[23,30] This contrasts with the significantly low frequency (10%) of RVO patients with the CC/GG genotype. The highest density genotype (TT/AA) was 12.5% in our normal control group, again very similar to the 4.8–19% reported in other studies.[23,30] Interestingly in the overwhelming majority of RVO patients, the GpIa/IIa status was the intermediate prothrombotic risk genotype CT/GA rather than the higher risk TT/AA. In the study by Matsubara *et al*,[24] diabetic patients with the TT/AA genotype had a 3.4-fold increase of retinopathy compared with the CC/GG genotype, with the CT/GA genotype having an intermediate risk. The expression of the various GpIa/IIa genotypes would be expected to follow simple Mendelian inheritance, but the relatively high expression in normal European populations of the low-risk CC/TT would suggest a protective effect leading to selection advantage over the prothrombotic TT/AA. Thus in our series, we were unable to show that TT/AA was more commonly associated with RVO than the intermediate-risk CT/GA because of a combination of a relatively low frequency for the TT/AA genotype in the general population and a relatively small study population.

There was no difference in the incidence of genetic mutations between those patients who suffered a single RVO compared to those who had recurrence. This may be because of patients commencing therapy for any underlying predisposing conditions, for example, hyperlipidaemia, and/or the institution of aspirin therapy, which would be expected to reduce the risk of recurrence. The finding that the incidence of mutations is similar in patients less than 50 years compared to older patients is of interest as cardiovascular risk factors are less prominent in this patient group.[33,34] The platelet GpIa/IIa status is therefore the first consistent abnormality to be identified in this patient group and adds support to the hypothesis of the possible aetiological significance of this abnormality.

It has been shown that both genetic and enviromental factors can interact additionally or synergistically to increase the risk of both arterial and venous thrombosis[36–39]. Likewise, our study shows that FVL, prothrombin gene mutations, and MTHFR can cause RVO when associated with another genetic abnormality, notably platelet GpIa/IIa polymorphisms. The frequencies of the various GpIa/IIa polymorphisms and the common thrombophilic conditions vary between ethnic groups, which may partly explain the conflicting results as to the role of FVL and MTHFR in RVO.[7,23,30,39] Other types of platelet glycoprotein polymorphisms exist. Larsson and Hillarp[40] recently reported, however, that another polymorphism of glycoprotein IIIa played no role in RVO.

In keeping with other studies, our results show that platelet GpIa/IIa status does not appear to be a significant risk factor for large vessel venous thrombosis, that is, DVT; in contrast is our finding that it may play a role in microvascular disease.[17,22] In a large cohort study in patients with RVO, we previously reported a

significantly higher MI rate and a trend towards a higher incidence of stroke in RVO patients even after other medical conditions, for example, hypertension, hyperlipidaemia, and treatment (eg aspirin), were taken into account.[41] Several studies have shown that polymorphisms of GpIa/IIa may be associated with large vessel arterial disease, and our identification that they also play a role in RVO suggests a common genetic link and may explain the higher risk of MI in RVO patients.[19–23]

In conclusion, our study suggests a major, possibly pivotal role for polymorphisms of the platelet glycoprotein receptor GpIa/IIa in the pathogenesis of RVO. Although larger studies allowing multivariate analysis will be required to confirm our findings, these data present for the first time a potential genetic marker that could have major implications for the aetiology and management of microvascular disease, in this case RVO.

Contributions

Chris Fegan and Paul Dodson designed the study, analysed the data, and wrote the paper. Paul Dodson and Jackie Farmer obtained ethical permission, blood samples, and data collection from RVO patients. Chris Fegan and Rod Johnson collected blood samples and data from the DVT group and normal controls. Jane Starczinski, Sarah Shigdar and Jenny Haynes performed all the molecular analysis. Greg Fegan performed the statistical analysis.

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