A specific allele of the histidine-rich glycoprotein (HRG) locus is linked with elevated plasma levels of HRG in a Dutch family with thrombosis

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Summary. Recent studies describe families with both elevated plasma HRG levels and thrombosis. In order to study the possibility that allelic variants of the HRG locus are associated with differences in HRG level, we studied linkage between HRG levels and a dinucleotide repeat polymorphism in a Dutch family which was selected on the presence of both thrombosis and elevated plasma HRG levels. No other known risk factors from thrombosis were found in this family. Linkage was calculated between the dinucleotide repeat and the HRG level assuming a population prevalence of elevated HRG of 5%. Two classes of HRG levels were defined by a mean and a variance: one class with normal HRG levels and a second class with high HRG levels. Using a mean HRG level of 99% for individuals with a normal HRG level and 145% for individuals with high HRG, a maximum lod score of 4.17 (odds in favour of linkage of 22 000 : 1) was found at a recombination fraction of 0, indicating linkage. Considering the pedigree, an association was found between the presence of a specific allele (no. 6) of the dinucleotide repeat polymorphism and plasma HRG levels. Family members carrying allele 6 were found to have higher HRG plasma levels compared with family members lacking allele 6 (149% v 109% respectively). We conclude that in this family, linkage is found between the HRG locus and the HRG level, and that a HRG gene coupled to allele 6 of the dinucleotide polymorphism is associated with elevated plasma HRG levels. No evidence was found for a causal relationship between elevated plasma HRG levels and thrombosis in this family.

Keywords: family, linkage, histidine-rich glycoprotein, HRG, thrombosis, polymorphism.

Histidine-rich glycoprotein (HRG) is a single-chain glycoprotein which is found in human plasma at a concentration of 100 µg/ml (Heimbürger et al, 1972). A pool of HRG is also stored in the α-granules of platelets at a concentration of about 0.4 µg/10⁸ platelets (Leung et al, 1983; Hoffmann et al, 1993). Plasma HRG is produced by the parenchymal cells of the liver (Hennis et al, 1991) and the source of platelet HRG is probably the megakaryocyte (Leung et al, 1983). Up to now, the exact physiological function of HRG is unknown, but it has been proposed that HRG can act as a modulator of coagulation and fibrinolysis (Koide, 1988). HRG has been shown to interact with a variety of haemostatic factors such as heparin (Koide et al, 1982), fibrinogen (Leung, 1986) and plasminogen (Lijnen et al, 1980). The ability of HRG to bind plasminogen is thought to affect fibrinolysis by reducing the amount of fibrin-bound plasminogen which is available for activation into plasmin. An elevation of the HRG level in plasma may therefore reduce the fibrinolytic potency.

Supporting evidence for this hypothesis is presented in several studies of patients with thrombosis (Samama et al, 1983; Engesser et al, 1988; Ehrenforth et al, 1994). In these patients a higher prevalence of elevated HRG levels has been observed. Moreover, in recent years six families have been described in which both thromboembolic disease and elevation of plasma HRG was observed (Falkon et al, 1992; Anglès-Cano et al, 1993; Castaman et al, 1993; Hoffmann et al, 1993; Engesser et al, 1987). All members of these families with a history of thrombosis had elevated levels of...
Fig 1. Pedigree of family. Filled symbols indicate elevated HRG plasma levels (>145%); dashed symbols indicate a history of thrombosis. Family members who were not available for investigation are indicated with N.A. The HRG level and the genotype with respect to the dinucleotide repeat are shown below each individual. The proband is indicated by an arrow.
HRG, although a few family members had elevated HRG levels and no history of thrombosis. However, up to now it is unknown whether the relationship between congenitally elevated HRG levels and thrombosis is causal or coincidental.

Recently, a twin study was presented in which genetic factors were found to explain 69% of the variance in plasma HRG levels (Boomsma et al, 1993). From this study it is clear that a familial elevation of HRG levels cannot be attributed to common environmental influences shared by family members, but has to be attributed to genetic influences shared by parents and children. Several genes, among them the HRG gene which is localized on chromosome 3q28-q29 (Hennis et al, 1994), may be responsible for this genetic influence. In some reports it has been speculated that the inherited elevated HRG levels find their cause in an abnormal HRG gene (Hoffmann et al, 1993; Schved et al, 1991), but up to now no association has been reported between elevated HRG levels and a specific allele of the HRG gene.

To investigate the role of the HRG locus in congenital elevation of HRG, we studied the inheritance pattern of a multi-allelic dinucleotide repeat polymorphism within the HRG gene (Hennis et al, 1994) in a Dutch family with both elevated HRG levels and thrombosis. Quantitative linkage analysis suggested involvement of the HRG locus. One allele of the highly informative dinucleotide repeat was found to be linked with high plasma HRG levels.

METHODS

Case report and family history. The proband with thromboembolic disease and elevated HRG came to our attention in a previous study (Engesser et al, 1988). The proband (II-18, see pedigree in Fig 1) was a 46-year-old man seen for episodes of recurrent thromboembolism; deep venous thrombosis (DVT) in the right leg and pulmonary embolism after groin rupture surgery at the age of 32; 1 year later DVT in the left leg after stopping oral anticoagulant therapy with coumarin. At the age of 35 a spontaneous thrombosis was diagnosed in the right leg. In all three episodes DVT was confirmed by phlebography.

The pedigree is shown in Fig 1. Persons with a history of thrombosis are indicated. Both parents of the patient suffered from DVT. The father (I-1) had DVT in the right leg at the age of 77. The mother (I-2) had DVT in the right leg at the age of 75. One of the proband's sisters (II-1) had recurrent DVT after an operation at the age of 40. A brother of the proband (II-3) had DVT in the right leg at the age of 29 after a trauma. One family member (II-14) who is related by marriage had postoperative DVT in a leg. Another family member (II-12) who is related by marriage was treated with percutaneous transluminal coronary angiography (PTCA).

Oral contraceptives were used by four females in generation III (11, 15, 17 and 18).

Blood collection. This study was approved by the Medical Ethical Committee of HGO-TNO and informed consent was obtained from patients and controls.

Blood was collected from family members by a single venipuncture into vacutainer tubes (Becton Dickinson, France) with sodium citrate as anticoagulant. Samples were kept on ice and centrifuged immediately at 3000g for 30 min at 4°C. Plasma was separated and stored at −80°C until use. The remaining blood cells and Buffy coat of the samples were used for isolation of genomic DNA.

Coagulation assays. Assays of activated partial thromboplastin time, prothrombin time, thrombin clotting time, fibrinogen (Clauss method) and factor VIII coagulant activity were performed according to established procedures. Coagulant activities of factor V and VII were measured by one-stage clotting assays, using factor V and VII deficient plasma and rabbit brain thromboplastin (Simplastin-plus, General Diagnostics, Morris Plains, U.S.A.). Protein S antigen was measured by a radioimmunoassay previously described by Bertina et al (1985). Antithrombin III activity was determined by a spectrophotometric assay (Coastet, Kabivitrum, Sweden). Von Willebrand factor antigen, factor II and factor X antigen, huparin cofactor antigen (Bertina et al, 1987) and protein C antigen (Bertina et al, 1982) were measured by Laurell rocket electroimmunoassay. The anticoagulant response to activated protein C (APC) was measured using Coastest® APC resistance (Chromogenix, Sweden). Protein C activity was determined using a new assay based on the measurement of coagulation times on a Coag-A-Mate® RA-4 (Organon Teknika, The Netherlands). The assay has been developed by Organon Teknika and will be available in the near future. The procedure was started by the reconstitution of a vial of Protac (Pentapharm, Switzerland) in 6 ml APTT-L (Platelin-L®, Organon Teknika) reagent (Protac concentration: 0.5 U/ml). The APTT/Protac solution was allowed to stand for 15 min at 0°C. A calibration curve was made by diluting Verily® Reference Plasma (Organon Teknika) 0, 5 and 10 times in Protein C depleted plasma. Plasma of patients was diluted 10 times in protein C depleted plasma. Curves containing 100 μl of the calibration solutions or the diluted samples were inserted into the RA-4 and the measurement was started. After a fixed heating time, which cannot be influenced, 125 μl of APTT/Protac solution was added via the Coag-A-Mate® RA-4. Following an incubation time of 220 s at 37°C, 100 μl CuCl2 solution (Platelin®, Organon Teknika) was added and coagulation times were registered. Protein S activity was determined with essentially the same procedure using however protein S deficient plasma, 125 μl CuCl2 and 100 μl of APTT/Protac.

Fibrinolysis assays. Plasminogen (Plg) activity was determined on a MLA 1000C (Medical Laboratory Automation, Pleasantville, New York, U.S.A.) using a chromogenic assay (Dade®, Baxter, Miami, U.S.A.). For calibration Dade® CoagCal® N (Lot no. 540.046, Baxter, U.S.A.) was used. The assigned value of the plasminogen level in this plasma is 106%. a2-antiplasmin activity was measured by a chromogenic assay according to Fribinger et al (1978). Tissue-type plasminogen activator (t-PA) activity was measured according to Verhelst et al (1982). Urokinase-type plasminogen activator (u-PA) antigen and activity were determined using an ELISA (Binnema et al, 1986) and a biological immunoassay (van Hinsbergh et al, 1990), respectively. Fibrinolytic activity of the intrinsic plasminogen activating system was
detected on fibrin plates, using euglobulin fractions from plasma, prepared in the presence of dextran sulphate, with or without the addition of antibodies against u-PA (Kluft et al., 1984). Plasminogen activator inhibitor (PAI) activity was measured according to Verheijen et al. (1984).

**Assay of histidine-rich glycoprotein.** Measurement of plasma HRG levels was performed by radial immunodiffusion (Mancini et al., 1965) as previously described (Boomsma et al., 1993). Using this method, HRG levels were determined in 126 healthy individuals. The mean HRG level was 99% (SD 20%) and the range 56–145%.

Electrophoretic mobility of HRG in the presence of Glu-plasminogen was studied by affinity crossed immunoelectrophoresis according to Kluft & Los (1988). Heparin binding was studied by affinity crossed immunoelectrophoresis with 100 IU heparin per ml (Thromboliquine, Organon Teknika, The Netherlands) and 1 mM CuCl2 in the first dimension (Hoffmann et al., 1993).

**Detection of polymorphisms.** Genomic DNA was obtained from freshly collected blood as described previously (Wijmenga et al., 1990). Genotypes with respect to the dinucleotide repeat were detected by use of the polymerase chain reaction (PCR) with radiolabelled nucleotides (Hennis et al., 1992), using primers HRG-CA2F: 5′ AAG CAG ACT TTG TCA TGG CAG TGC 3′ and HRG-CA2R: 5′ TTG CAC TCC TTT CCC CAG TGG 3′ (Isogen Bioscience, Amsterdam, The Netherlands). Individual genotypes were determined using a standard set of repeats of known length (Fig. 2). The repeat is localized in the last intron of the HRG gene (Hennis et al., 1994).

**Linkage.** Linkage was carried out using the MLINK option of the LINKAGE computer program package, version 5.03 (Lathrop & Lalouel, 1988). Linkage was calculated between the phenotype 'HRG level' and the dinucleotide repeat that is located in the last intron of the HRG gene. HRG levels were considered as a quantitative phenotype under the assumption that elevated HRG is a monogenic dominantly inherited trait (Ott, 1991). Two classes were distinguished: one with normal HRG levels (unaffected) and a second with high HRG levels (affected). In this approach the two classes were defined by a mean HRG level and a variance. For the class with normal HRG levels, a mean HRG of 99% and a variance of 400 was used in all calculations. This mean level and variance were derived from a control group of 126 volunteers. For the class with high HRG levels, lod scores were calculated for mean levels of 135%, 145%, 155% and 165%. The variance of HRG levels was assumed to be equal for both classes. The population frequency of high HRG levels was assumed to be 0.05. No corrections for possible age effects on the HRG level have been implemented in the linkage analysis.

**RESULTS**

**Plasma levels**

In order to exclude the possibility that the apparent familial thrombophilia was due to known or suspected disorders of fibrin formation and fibrinolysis, we studied the proband extensively on coagulation and fibrinolysis parameters (Table I). No abnormalities were found in the coagulation assays taking the intensity of oral anticoagulant treatment into account. Protein C, protein S activities and resistance to activated protein C were not determined in the proband who was on stable anticoagulant treatment. To exclude familial type II protein C or protein S deficiency or resistance to activated protein C, these parameters were measured in the spouse, several brothers and sisters and both the children of the proband. No abnormalities were found in the family members investigated. Fibrinolysis parameters were found to be normal in the proband. A minor elevation of u-PA activity was observed.

A persistently elevated level of HRG was found in the proband (161%, and 154% 3 years later). In nine other family members we also found high levels of HRG up to 209% (see pedigree in Fig 1). The mean HRG level of all family members was 124%, which was high compared to the mean level found in 126 healthy individuals (99%, SD 20%;

| Table I. Coagulation and fibrinolysis parameters in the proband’s plasma. |
|-----------------|-----------------|
|                 | Proband*        | Normal range† |
| Clotting times  |                 |                |
| APTT (s)        | 29.4            | 28.0–39.2‡     |
| Prothrombin time (s) | 17.0      | 17.0–19.0‡     |
| Thrombin clotting time (s) | 16.1 | 14.3–18.5‡     |
| Coagulation factors |             |                |
| Fibrinogen (mg/ml) | 2.2          | 1.8–4.9        |
| Factor II antigen (%) | 57          | 46–77‡         |
| Factor V activity (%) | 100         | 52–124         |
| Factor VII activity (%) | 28          | 60–157         |
| Factor VIII coagulant activity (%) | 172  | 50–200        |
| Factor X antigen (%) | 61           | 31–76‡         |
| Von Willebrand factor antigen (%) | 165   | 31–216         |
| Antithrombin III activity (%) | 91     | 75–120         |
| Heparin cofactor II antigen (%) | 115  | 61–185         |
| Protein C antigen (%) | 55          | 40–70‡         |
| Protein S antigen (%) | 60         | 36–66‡         |
| Fibrinolysis factors |             |                |
| Plasminogen activity (%) | 113   | 70–140         |
| t-PA activity (mIU/ml) | 13.2    | >0             |
| u-PA activity (ng/ml) | 3.5      | 1.3–3.1        |
| u-PA antigen (ng/ml) | 4.3      | 1.7–6.2        |
| Factor XII-dependent plasminogen activator activity (%) | 95   | 76–158         |
| Plasminogen activator inhibition activity (IU/ml) | 6.4 | 1.7–34.0        |
| α2-antiplasmin activity (%) | 95     | 80–120         |
| HRG antigen (%) | 161/154‡      | 56–145         |

* Oral anticoagulant treatment at the time of investigation with an intensity of 2·07 International Normalized Ratio.
† Normal ranges were established in at least 42 healthy members of the laboratory staff. The normal range of HRG was established in 126 healthy volunteers.
‡ Values of 23 individuals on stable anticoagulant treatment with a mean of 2·4 International Normalized Ratio, which is slightly higher compared to the intensity of anticoagulation in the proband.
§ A persistent elevated HRG antigen was confirmed after 3 years.
Elevated HRG Levels associated with a Specific HRG Allele

range 56–145%). Possible effects of assortative mating have been excluded in a previous parent–twin study on the heritability of HRG levels (Boomsma et al., 1993). Four women in generation III (III-11, III-15, III-17 and III-18) used oral contraceptives which may lead to a decrease in HRG level of 20–25% (Jespersen et al., 1990). One of these women (III-17) had an elevated HRG. The other three women had normal HRG, and this would not become elevated when a correction of 25% was applied. No corrections were therefore made for the usage of oral contraceptives.

A normal plasminogen and heparin binding of HRG was observed in an affinity crossed immunoelectrophoresis assay with plasma of the proband (data not shown).

Dinucleotide repeat genotypes
The genotype with respect to dinucleotide repeat was determined in all family members. Eight different allele types were found and heterozygosity was observed in 30/34 family members (see pedigree in Fig 1). A standard set of allele types of the dinucleotide repeat is shown in Fig 2. Only in one marriage was information lost due to similar alleles of the dinucleotide repeat in father and mother (II-9 and II-10). No evidence for recombinations or mutations was found within this pedigree.

Linkage
Linkage was performed regarding HRG levels as a quantitative trait with a bimodal distribution caused by two classes. Lod scores and corresponding recombination fractions were calculated assuming a mean level of 99% for the class with normal HRG levels and different mean HRG levels (135%, 145%, 155% and 165%) for the class with high HRG levels.

Maximum lodscores for all analyses were found at $\Theta = 0$ indicating complete linkage. A maximum lod score of 4.65 at $\Theta = 0$, representing odds in favour for linkage of 46 000:1, was found at the lowest mean level (135%) for the high-level class. When the mean of the high-level class was set at 145% which was the highest HRG level of the normal range, a lod score of 4.17 was found (odds in favour for linkage 22 000:1). Higher mean values of 155% and 165% both gave lod scores of 2.99 which represent odds in favour of linkage of 1000:1.

In all calculations presented above, a population prevalence of elevated HRG levels of 5% was assumed. However, lod scores did not show substantial changes when the population prevalence was set at 10% or 1% (data not shown).

Dinucleotide repeat genotype and HRG level
Elevated levels of HRG were frequently found in association with family members who carry allele 6 of the dinucleotide repeat polymorphism, indicating that high HRG levels are linked to this allele. HRG levels of individuals with allele 6 were on average higher (149%, $n = 13$) than of individuals without allele 6 (109%, $n = 25$). The mean HRG level of all individuals was 124% ($n = 34$).

The genotype was not in all cases predictive for an elevated HRG level (see pedigree in Fig 1). In five family members a normal HRG level was found in combination with allele 6 (III-2, III-8, III-9, III-20, III-21). However, three out of these five individuals did have a HRG level which is above the mean HRG level in the family. An elevated HRG level was found in two individuals who did not carry allele 6: a father (II-12) who was related by marriage and his daughter (III-17).
HRG levels and thrombosis

Of the six family members with a history of thrombosis, three members had an elevated HRG level. One individual with a history of thrombosis was related by marriage (II-15) and had no elevated HRG. Both grandparents had thrombosis but could not be included in the present analysis. Elevated HRG levels were also found in seven family members without a history of thrombosis.

DISCUSSION

In this paper we describe a family with familial thrombosis in which no known risk factors for thromboembolic disease could be found. The only abnormality observed was a high level of HRG. By investigating this family, high levels of plasma HRG were found in 10/34 family members distributed over two generations, suggesting a hereditary nature for this trait.

To investigate the possibility that specific HRG gene variants are associated with elevated plasma HRG levels in our family, we studied the inheritance pattern of a multi-allelic dinucleotide repeat which is located in the intron between the last two exons of the HRG gene (Hennis et al., 1994). Due to the large number of alleles and a high degree of heterozygosity, little information is lost in the pedigree.

Linkage was calculated between the dinucleotide repeat and plasma HRG levels regarding the HRG level as a quantitative trait and assuming a bimodal distribution of HRG levels. Two classes, one with normal levels of HRG (unaffected) and a second with high levels of HRG (affected), were defined by a mean and a variance. Using this approach, lod scores tended to decrease when the mean value for the class with high HRG levels was increased. This can be explained by a reduction of the overlap between the distributions of the two classes. The higher the mean HRG level of the high-level class was chosen, the more family members have a possibility to be assigned to the normal-level class. These individuals are then considered as recombinants which leads to lower lod scores.

By convention a lod score of 3 (odds in favour of linkage of 1000:1) is taken as a threshold for linkage. In the present family we found a maximum lod score of 4-65 and a minimum of 2-99, both at zero recombination, presenting substantial evidence for linkage. This may indicate a significant effect of the HRG locus on plasma HRG levels in general, although it may also be a unique effect in this family. In all calculations a population prevalence of elevated HRG levels of 5% was assumed (Boomsma et al., 1993). However, lod scores did not show substantial changes when the population prevalence was set at 10% or 1%.

The majority of the elevated HRG levels appeared to be coupled to allele 6 of the polymorphism (Fig 1). A marked difference was found between the average HRG level of family members with allele 6 and members without allele 6 (149% v 109%). This result suggests a specific effect of the HRG gene which is coupled to allele 6, on the HRG level. The allele frequency of allele 6 in a group of 130 healthy volunteers is 1-5%. In this group there is no indication that allele 6 is coupled to elevated HRG levels (Hennis, unpublished observations). Therefore it seems reasonable to assume that allele 6 itself is not related with raised HRG levels and that the association between allele 6 and elevated HRG levels is most likely to be purely by linkage within this family.

Not all family members with allele 6 do have elevated HRG levels. It is possible that the lower levels of HRG in these family members are due to neutralizing effects caused by other HRG alleles, assuming that a multiallelic HRG locus is present. In one father (who is married into the pedigree) and his daughter, an elevated HRG level is found in the absence of allele 6, suggesting that the HRG gene associated with elevation of HRG may also be coupled with other alleles of the dinucleotide repeat.

Linkage cannot discriminate between major and minor genes (i.e. the genetic background) that are involved in a given trait. It is therefore not possible to exclude a polygenic model. This may either be a model for a multi-allelic HRG locus or a model involving different loci. In a recent parent–twin study (Boomsma et al., 1993) 69% of the variance in HRG level could be ascribed to genetic background. In such a study design the dinucleotide polymorphism could be used to determine the contribution of the HRG locus to the heritability.

In theory, elevation of plasma HRG levels can be caused by an abnormality in the promoter region or elsewhere in the gene causing over-expression of the protein. An increased level can also be due to a deviating protein structure resulting in either increased synthesis or decreased clearance. In the investigated family no indication was found for the presence of an abnormal HRG molecule. Both the binding of heparin and plasminogen appeared to be normal in an affinity crossed immunoelectrophoresis assay. As yet, nothing has been reported about abnormal forms or polymorphisms of the HRG molecule in relation to either high HRG levels or thrombosis.

Another possibility is that the elevation of HRG is age dependent. In this family a statistically significant correlation (r = 0-35, P < 0-05) was observed between age and HRG level. A comparable age-dependent increase with an even stronger correlation was found in a family described by Hoffmann et al. (1993). Recently, it was also reported that parents have higher HRG levels than their children (Boomsma et al., 1993), but thus far no correlation between age and HRG level has been found in groups of unrelated healthy individuals (Hoffmann et al., 1993). Age may have consequences for linkage, because the overlap between the classes of normal and high HRG levels may be greater in the younger generation leading to more false recombinants. However, as the magnitude of the age effect is unknown, no corrections for age have been implemented in the linkage analysis of this family.

In a previous report it is hypothesized that elevated HRG levels may contribute to a prothrombotic state by reducing the availability of plasminogen which can be activated into plasmin (Lijnen et al., 1980). Elevated HRG levels are found in three out of six family members with thrombosis. However, due to the small number of family members with
a history of thromboembolic disease, no conclusions can be based on this particular family with respect to a causal relationship between thrombosis and elevation of HRG (see also pedigree in Fig 1). To study this relationship further, more families have to be included in future studies. In addition, characterization of the HRG gene and of the function of HRG in the haemostatic system are needed to understand the possible contribution of elevated HRG to the prothrombotic state. It is therefore important to screen the HRG gene for functional mutations or polymorphisms in families with high HRG levels.

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