Histidine-rich Glycoprotein (HRG) Tokushima 2: Novel HRG Deficiency, Molecular and Cellular Characterization

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Summary
The proband, a 76-year-old woman, suffered from dural arteriovenous fistula. Her plasma histidine-rich glycoprotein (HRG) level was 50% of the normal level. A low level of plasma HRG was also found in her third daughter. A single nucleotide substitution of T to C was identified in her third daughter, converting Cys223 to Arg in the second cystatin-like domain. The same mutation was also identified in her third daughter, but not in the other four family members having normal HRG levels or in 50 unrelated healthy Japanese individuals. Expression studies in BHK cells showed that substantial intracellular degradation of the mutant occurred and only about 40% of the recombinant HRG mutant was secreted. These results indicate that congenital HRG deficiency caused by a substitution of Cys223 to Arg is hereditary in this family.

Introduction
Human histidine-rich glycoprotein (HRG) is a single-chain glycoprotein with a molecular weight of 67,000. It is composed of 507 amino acids and belongs to the cystatin superfamily that includes cystatin SN, SA and C, and kininogen (1, 2). The human HRG gene spans approximately 11 kb on chromosome 3q28-q29 and consists of seven exons and six introns (3, 4). HRG circulates in blood at relatively high concentration (~100 mg/L) (5) with a half-life of three days (6). Platelets store HRG in the α-granules and secrete HRG upon thrombin activation (5). HRG has been shown to interact with heme (7), metal ions (7), heparin (8), fibrinogen (9), factor XIIIa (10), plasminogen (11), thrombomodulin (12), several complement factors (13), activated human platelets (14), and T lymphocytes (15). The physiological function of HRG has not been established (16, 17), but in vitro observations suggest that it plays a role in vivo in metal homeostasis, immunoregulation, and modulation of blood coagulation and fibrinolysis.

In 1993, we discovered the first case of congenital HRG deficiency (HRG Tokushima). The proband, a 43-year-old woman, suffered from cerebral sinus thrombosis (18). Her hemostatic tests were all normal except for plasma HRG level, which was only 21% of the normal level. Low levels of plasma HRG were also found in four family members. Recently, we identified a single heterozygous mutation of Gly85 to Glu for congenital HRG deficiency in this family (19). In 1996, Sout et al. reported a second family of this deficiency (20). The patient developed pulmonary embolism on two occasions at the age of 36, while her father had thrombosis in the central artery of the retina at the age of 59. However, the genetic abnormality in this family is still unclear (B. Henri, personal communication). In this report, we describe a third case of congenital HRG deficiency.

Methods
Hemostatic studies. The platelet count, prothrombin time, activated partial thromboplastin time (APTT) and plasma fibrinogen level were measured by standard techniques. The serum concentration of fibrinogen-fibrin degradation products (FDP) was assayed by latex photometric immunoassay using LPIA-1 FDP-E (Teikoku Hormone Mfg., Tokyo, Japan). Anticardiolipin antibody was detected with an anticardiolipin-β2-glycoprotein I kit YAMASA ELISA (Yamasa Shoyu, Chiba, Japan). Antithrombin activity, heparin cofactor II (HCII) activity and plasminogen activity were determined by chromogenic methods using Testzym ATIII-2 (Daiichi Kagaku, Tokyo, Japan), Stachrom HCII (Diagnostica Stago, Asnières, France) and Testzym PLG-2 (Daiichi Kagaku), respectively. Protein C activity and protein S activity were estimated by clotting time methods using Staclot protein C and Staclot protein S (Diagnostica Stago), respectively. Activated protein C (APC) resistance was determined by the APTT-based assay using COATEST APC Resistance (Chromogenix AB, Mölndal, Sweden). Plasma HRG concentration was assayed by Laurell’s method (21) with rabbit anti-human HRG (Diagnostica Stago).

Polymerase chain reaction (PCR) and DNA sequence analysis. Oligonucleotide primers were synthesized based on the intron sequences approximately 50 bp apart from the intron/exon boundaries of the HRG gene (3) using the 380B DNA Synthesizer (Applied Biosystems, Foster City, CA). Exon 7 was divided into five segments separately amplified using the primers synthesized similarly as before. The sequences of all primers used for PCR were the same as those used before (19). The PCR mixtures contained PCR buffer (10 mmol/L Tris hydrochloride, pH 8.4, 50 mmol/L KCl, 1.5 mmol/L MgCl2, and 0.01% gelatin), 1 µg genomic DNA, and 2 U Taq polymerase (TaKaRa Ex Taq, TaKaRa, Shiga, Japan). Amplification was performed as follows: denaturation at 94° C for 1 min, annealing at 55° C for 1 min, and extension reaction at 72° C for 1.5 min for 35 cycles. The amplified DNA fragments were directly
subcloned into pCR2.1 vector supplied with the TA Cloning System kit (Invitrogen, San Diego, CA). The DNA sequence was determined with a Dye Primer Cycle Sequencing FS Ready Reaction kit (Applied Biosystems) on an ABI PRISM 377 sequencer (Applied Biosystems).

Allele-specific oligonucleotide (ASO) hybridization analysis. ASO probes employed in this study contained the normal sequence 5'-GTCATAAACGGAAGTCT-3' and the mutant sequence 5'-GTCATAAACGGAAGTCT-3' (underlines indicate the base change). Five microliters of amplified PCR fragments of exon 6 were spotted onto a Hybond-N+ membrane (Amersham Pharmacia Biotech) and air dried, and hybridized at 46°C with normal ASO or mutant ASO probe, then labeled using ECL 3'-oligolabelling and detection systems (Amersham Pharmacia Biotech) according to the manufacturer's recommended protocol. The hybridized membrane was exposed to Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY) at room temperature for 3 min with an intensifying screen. Fifty unrelated normal individuals were studied to analyze the genotype of exon 6 in the HRG gene.

Construction of expression vector. A region of nucleotides 118 to 2067 of human HRG cDNA (HHRG3) was used for the expression experiment. Nucleotide 118 is just four bases upstream from the initiation codon ATG, and this cDNA was subcloned in pUC19 plasmid. A BamHI site was introduced into this DNA at the 3' end by PCR using a primer, 5'-TTGATCATCTTCGAC-3'. This PCR product was used as one of the primers for successive second-round PCR. The other was a universal -40 primer. The whole cDNA fragment carrying the mutation thus amplified was digested with EcoRI I and BamHI I and inserted into ZMB3 expression vector. To avoid incorporation of an unexpected mutation, the EcoRI I/Bal I fragment (nucleotide 118 to 1411) was replaced by that of the original clone. The 3' region from the Bal I site to the BamHI I site was sequenced to verify the absence of mutation. The cDNA carrying the mutation found in the proband was amplified with the above-mentioned primer and the mutated primer, 5'-ATAAACCCTGGAAGTCTTCCGCAC-3'. This PCR product was used as one of the primers for successive second-round PCR. The other was a universal -40 primer. The whole cDNA fragment carrying the mutation thus amplified was digested with EcoRI I and Hind II, and subcloned into pUC19 plasmid to verify incorporation of the mutation without any other unexpected replacements. The whole cDNA was then constructed on pUC19 plasmid, released by EcoRI I and BamHI I, and ligated into ZMB3 vector.

Cell culture and pulse-chase experiments. To examine secretion of the Tokushima 2-type HRG mutant, pulse-chase experiments were performed using stably expressing BHK cells as described previously (19). Briefly, about 5 x 10^6 cells were cultured overnight under the standard conditions and then starved for methionine and cysteine for 30 min before pulse-labeling with 50 μCi EXPRESS2/53S(NEN-DuPont, Tokyo, Japan). After 1 h, they were washed with phosphate-buffered saline and culture media containing 2 mmol/L each of methionine and cysteine, and chased in the same media for defined periods. At selected time intervals, culture media were harvested and cells were lysed. The labeled HRG was immunoprecipitated using affinity-purified rabbit anti-human HRG IgG and ZYSORBIN (ZYMED Laboratories, Inc., So. San Francisco, CA). After washing, immunoadsorbed proteins were dissociated by heating at 85°C for 5 min, and then electrophoresed on 8% polyacrylamide gel in the presence of SDS and by heating at 85°C for 5 min, and then electrophoresed on 8% polyacrylamide gel in the presence of SDS and by heating at 85°C for 5 min, and then electrophoresed on 8% polyacrylamide gel in the presence of SDS and by heating at 85°C for 5 min, and then electrophoresed on 8% polyacrylamide gel in the presence of SDS and by heating at 85°C for 5 min, and then electrophoresed on 8% polyacrylamide gel in the presence of SDS and by heating at 85°C for 5 min, and then electrophoresed on 8% polyacrylamide gel in the presence of SDS and by heating at 85°C for 5 min, and then electrophoresed on 8% polyacrylamide gel in the presence of SDS and

Results

Case history. The proband (II-6 in Fig. 1) was born in Tokushima Prefecture in 1919. Her parents were unrelated. Her father (I-1) died of hepatitis, while her mother (I-2), elder sister (II-1) and second elder brother (II-4) died of unknown cause and without clinical details. Her eldest brother (II-2) and first younger brother (II-7) were killed in action, while her second younger brother (II-8) died of lung cancer. The proband had been treated for hypertension since 1970, hypothyroidism since 1977, and coronary insufficiency since 1987. She began to have right frontal headaches in July, 1995, complained of diplopia on September 18, 1995, and entered our hospital on October 24, 1995. Exophthalmos was present with conjunctival chemosis and paralysis of abducens movement of both eyes. Angiographic examination of her brain revealed a dural arteriovenous fistula (DAVF) (Fig. 2). She underwent transarterial embolization with coils and subsequently radiation therapy of 20 Gy. All of her symptoms gradually resolved.

Hemostatic studies. The proband’s platelet count, prothrombin time, APTT, plasma fibrinogen level and serum FDP concentration were all within normal ranges (Table 1). Anticardiolipin antibody was not detected in her serum. Her anticardiolipin activity, HCII activity, protein C activity, protein S activity, APC resistance-ratio and plasminogen activity were normal, but her plasma HRG level was 50% of the normal

Table 1 Hemostatic findings in the proband

<table>
<thead>
<tr>
<th>Studies</th>
<th>Proband</th>
<th>Normal range</th>
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<tbody>
<tr>
<td>Platelet count ( x 10^9/μl)</td>
<td>16.1</td>
<td>15-35</td>
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<tr>
<td>Prothrombin time (s)</td>
<td>9.9</td>
<td>9.8-11.3</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>34.0</td>
<td>29-42</td>
</tr>
<tr>
<td>Fibrinogen (mg/dl)</td>
<td>361</td>
<td>143-328</td>
</tr>
<tr>
<td>FDP-E (ng/ml)</td>
<td>84</td>
<td>&lt;100</td>
</tr>
<tr>
<td>Antithrombin activity (%)</td>
<td>91</td>
<td>80-130</td>
</tr>
<tr>
<td>Heparin cofactor II activity (%)</td>
<td>82</td>
<td>65-140</td>
</tr>
<tr>
<td>Protein C activity (%)</td>
<td>103</td>
<td>80-112</td>
</tr>
<tr>
<td>Protein S activity (%)</td>
<td>100</td>
<td>66-140</td>
</tr>
<tr>
<td>APC resistance-ratio</td>
<td>2.6</td>
<td>&gt;2</td>
</tr>
<tr>
<td>Plasminogen activity (%)</td>
<td>115</td>
<td>74-123</td>
</tr>
<tr>
<td>Anticardiolipin antibody (U/ml)</td>
<td>&lt;1.3</td>
<td>&lt;1.3</td>
</tr>
<tr>
<td>HRG antigen (%)</td>
<td>50</td>
<td>58-161</td>
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</tbody>
</table>
The level of 109.5 ± 51.5% (mean ± 2 SD). The concentrations of plasma HRG in five members of the family of the proband were also determined. The proband’s third daughter had a low plasma HRG level (Table 2). The proband and her third daughter, however, had no clinical features suggestive of factors that might have caused increased degradation or reduced synthesis of plasma HRG (22). These results suggest that congenital HRG deficiency is inheritary in this family.

**DNA sequencing.** To elucidate the genetic basis of congenital HRG deficiency, we examined the nucleotide sequences of all seven exons of the proband’s DNA. Each exon was amplified by PCR, subcloned into pCR2.1 vector, and transfected into INVINV/H9251/F’ cells. At least ten independent clones were isolated for each exon and sequenced. One single-base substitution was found in exon 6, and no substitution was found in the remaining exons. The amino acid residues of five polymorphic sites reported by Hennis et al. (23, 24) were identified as Ile162, Pro186, His322, Arg430, and Asn475. These all agree with those originally reported by Koide et al. (2), and no other polymorphisms were found in the proband’s HRG gene. A single T to C substitution at nucleotide position 11,438, resulting in a change of Cys223 to Arg in the second cystatin-like domain, was found in four of 15 different clones analyzed, while 11 clones contained the normal sequence (Fig. 3). These results indicate that the proband carries a heterozygous deficiency.

**ASO hybridization analysis.** To eliminate the possibility that the mutation found in the proband was a common polymorphism, we performed ASO hybridization analysis of the amplified PCR fragments of exon 6 from the proband, her family members and 50 unrelated normal individuals. The base-pair change (T/L50478C) was found only in the proband and her third daughter and was not present in unaffected family members (Fig. 4). The mutation was not found in any of the 50 normal individuals tested (data not shown), suggesting that the identified mutation is not a common polymorphism.

**Secretion of wild-type and Tokushima 2-type HRG expressed in stable BHK cells.** To elucidate if the mutation in HRG Tokushima 2 is responsible for the secretion defect of the mutant protein in the proband, we performed pulse-chase experiments of Tokushima 2-type HRG. (Left panel) Stably expressing BHK cells were pulse-labeled for 1 h and chased for 0, 0.5, 1, 2, and 4 h. Labeled HRG in the cell extracts and culture media were immunoprecipitated and analyzed on SDS-PAGE. (Right panel) Kinetic analysis of wild-type and C223R-type HRG. Solid circle shows the amount of HRG in culture media, open circle shows that in the cell extracts, and broken line shows a total amount of HRG.
HRG using stably expressing BHK cells, and compared the results with those obtained for wild-type HRG. Both wild-type HRG and Tokushima 2-type HRG were detected as a band of 62 kD and 72 kD in the cell extracts and culture medium, respectively. They were synthesized at nearly equal levels in BHK cells as detected in cell extracts at time 0 of the chase. During the chase period, wild-type HRG was secreted into the culture medium, and essentially no HRG band was detected in the 2-h chased cell extracts. However, the total amount of radioactivity was maintained during the chase period. In contrast, only a small amount of Tokushima 2-type HRG was secreted into the culture medium even after an 4-h chase, and a decrease in total radioactivity was observed. This suggests that intracellular degradation of the mutant HRG was occurring. The time to 50% disappearance of total radioactivity from the cell extracts was estimated to be 2 h.

Discussion

Here, we reported the third case of congenital HRG deficiency. The proband and her third daughter were heterozygous for this deficiency. The proband suffered from DAVF, but her third daughter had no symptom. The genetic cause for this case was a single missence mutation (T to C at nucleotide position 11,438 in exon 6) leading to a mutation of Cys223 to Arg in the second cystatin-like domain on the HRG molecule. Since this mutation was different from that of HRG Tokushima (19), we designated this case as HRG Tokushima 2. This is the second report on the molecular and cellular basis of congenital HRG deficiency.

DAVs are classified into three types: idiopathic, posttraumatic, and, very rarely, congenital. DAVF in the proband of HRG Tokushima 2 was idiopathic. The idiopathic type of DAVF tends to occur mainly, but not exclusively, in women over 45 years of age. It is generally agreed that it occurs at sites of previous thrombosis (25, 26). On the other hand, congenital HRG deficiency is probably one of inherited thrombotic disorders (27). A causal relationship between congenital HRG deficiency and thrombosis has not been established by only three cases (18, 20), but it is supported by the recent proposals that the function of HRG is pro-fibrinolytic rather than anti-fibrinolytic (28, 29). Therefore, association of congenital HRG deficiency and DAVF in the proband of HRG Tokushima 2 may not merely be incidental.

Cys223 and its surrounding sequence in HRG are rather conserved among species, including human (2), bovine (30), rabbit (31), rat 1 and 2, and mouse (Wakabayashi et al., manuscript in preparation) (Fig. 6). This may suggest that Cys223 is indispensable for correct folding of HRG. Cys223 forms a disulfide bond with Cys200 in the second cystatin-like domain. Cys plays a special role, since it can form cross-bridges (cystines) between different parts of the main chain by bonding to other Cys residues. Therefore, it is conceivable that Cys223 to Arg mutation caused a misfolding of HRG, leading intracellular degradation. Thus, the low plasma levels of HRG observed in the proband and her third daughter are likely to be resulted from intracellular degradation of the mutant HRG. This pathogenesis is fundamentally the same as that of HRG Tokushima (19). We suggested that not only endoplasmic reticulum (ER)-membrane anchoring proteins such as cystic fibroblast transmembrane conductance regulator (CFTR) (32), MHC class I heavy chain (33) and glycosylphosphatidylinositol (GPI)-linked protein (34), but typical secretory proteins, such as α1-antitrypsin (35), antithrombin (36) and protein C (37), are degraded by proteasome through the ER-associated quality control mechanism in the cell. To elucidate this in HRG Tokushima and Tokushima 2, cellular experiments are underway.

Acknowledgments

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References


