Heparin-Induced Thrombocytopenia: New Insights Into the Impact of the FcγRIIa-R-H131 Polymorphism

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Heparin-induced thrombocytopenia (HIT), a severe complication of heparin treatment, can be associated with new thrombotic complications. HIT antibodies activate platelets via the platelet Fcγ-receptor (FcγRIIa), which carries a functionally relevant polymorphism (FcγRIIa-R-H131). The effect of this polymorphism on the clinical manifestations of HIT is controversial. We determined prospectively the FcγRIIa-R-H131 genotypes in 389 HIT patients, in 351 patients with thrombocytopenia or thrombosis due to causes other than HIT and without detectable HIT antibodies, and in 256 healthy blood donors. For this purpose, a novel nested sequence-specific primer-polymerase chain reaction (SSP-PCR) was developed. FcγRIIa-R/R131 was found to be over-represented in the HIT patients (27%) compared with the control groups (non-HIT patients [21%] and blood donors [20%]). In a subgroup of 122 well-characterized HIT patients, the genotype distribution in patients presenting with thrombocytopenia only was compared with that of patients who developed thromboembolic complications. The frequency of FcγRIIa-R/R131 among patients with thrombotic events was significantly elevated (37% v 17%; P = .036). Our results indicate that genotype distribution can be correlated to the clinical outcome of patients with HIT. We speculate that the reduced clearance of immune complexes in patients with the FcγRIIa-R/R131 allele causes prolonged activation of endothelial cells and platelets, thus increasing the risk for thrombotic complications.

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MATERIALS AND METHODS

Patients and controls. Patients (n = 389) with clinical symptoms of HIT and heparin-dependent antibodies as determined by the heparin-induced platelet activation (HIPA) test15,16 and/or the HIT antibodies and with healthy blood donors. Additionally, we performed a subanalysis to assess the occurrence of the FcγRIIa-R-H131 polymorphism in HIT patients with isolated thrombocytopenia and in HIT patients presenting with TECs during heparin administration.

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subanalysis of these well-characterized patients was performed to determine the correlation between the \( \text{FcRRIa-R-H131} \) genotypes and the manifestation of TECs in HIT.

**HITa test.** Platelet-rich plasma (PRP) was prepared from citrated blood (1.6 vol adenine-citrate-dextrose [ACD] and 8.4 vol blood) from normal blood donors (with 10 medication-free days). PRP was acetylated by the addition of 111 \( \mu \text{mol/L} \) ACD, and 5 \( \mu \text{U/mL} \) apyrase (grade III; Sigma, Munich, Germany) were added. After centrifugation (7 minutes at 650g), the supernatant was discarded and platelets were carefully resuspended in 5 mL washing buffer (tyrode buffer containing 2.5 \( \mu \text{mol/L} \) apyrase and 1.0 \( \mu \text{mol/L} \) hirudin [Pentapharm, Basel, Switzerland], adjusted to pH 6.3 with HCl). The platelets were incubated in sealed tubes (15 minutes at 37°C), centrifuged (7 minutes at 650g), and resuspended in 1 mL suspension buffer (tyrode buffer containing 0.002 mol/L MgCl\(_2\) and 0.002 mol/L CaCl\(_2\), adjusted to pH 7.2 with HCl). The suspension was adjusted to 300,000 to 400,000 platelets/mL and incubated in a sealed tube (45 minutes at 37°C) before use. Heat-inactivated (56°C for 30 minutes) patient serum (20 \( \mu \text{L} \)) was dispensed in a microtiter plate (Greiner, Nürtineng, Germany). For intra-assay negative control, parallel samples are mixed with a high concentration of heparin (final concentration, 100 \( \mu \text{U/mL} \)). This procedure disrupts heparin-PF\(_4\) complexes and detects antibodies that bind independently of the heparin concentration. Platelet suspension (75 \( \mu \text{L} \)) is added to all samples and, finally, the low concentration heparin (final concentration, 0.2 \( \mu \text{mol/L} \)) is added to allow PF\(_4\)/heparin complex formation. The microtiter plate was incubated (45 minutes at room temperature) on a magnetic stirrer (1,000 rpm) with two steel spheres (2 mm diameter; SKF, Schweinfurt, Germany). The transparency of the suspension was assessed using an indirect light source every 5 minutes. The patient sera was considered positive for HIT antibodies if the suspension became transparent due to platelet aggregation with 0.2 U/mL heparin but not with 100 U/mL heparin.

**Primer design.** To genotype the \( \text{FcRRIa-R-H131} \) polymorphism, a new nested sequence-specific primer-polymerase chain reaction (SSP-PCR) method was developed. After the first PCR amplification, using primer pair P52 and P63,\(^7\) the product obtained was reamplified using sequence-specific primers. The sequence-specific sense primers P5G (specific for G507) and P4A (specific for A507) are located at the polymorphic site on exon 4, and a common antisense primer, P13, is located on intron 4 (Fig 1). Both exonic primers were constructed based on the published \( \text{FcRRIa} \) cDNA sequence.\(^8\) To increase the specificity, two mismatch bases (T instead of C) were introduced at positions 502 and 504 in both primers. Because no data on the intronic sequences of \( \text{FcRRIa} \) were available, we determined the nucleotide sequence of intron 4 by PCR using genomic DNA. After 30 amplification cycles with primers P63 and P52, a 1,000-bp product was isolated. Sequence analysis of this product identified an 800-bp intron with conserved nucleotide sequences. To validate the inter-assay reproducibility, an allelic ladder was generated using primer pair P63 and P52, and a 1,000-bp product was isolated. Sequence analysis of this 1,000-bp product confirmed the conservation of the nucleotide sequences.

**Nested SSP-PCR.** DNA was isolated from EDTA-anticoagulated peripheral blood using QIAamp blood kits (Qiagen, Hilden, Germany). One hundred nanograms of genomic DNA was added to 100 \( \mu \text{L} \) reaction mix containing 10 mmol/L Tris (pH 8.0), 50 mmol/L KCl, 2.75 mmol/L MgCl\(_2\), 0.25 mmol/L of each dNTP, 100 \( \mu \text{g/mL} \) bovine serum albumin (BSA), 0.1 mmol/L each of P63 (5'-CTA GCC GCT CAC CAC TCC TC) and P52 (5'-GAA GAG CTG CCA CCC ATG CG) primers, 20 mmol/L each of CRP I and CRP II primers, and 1 U of AmpliTaq (Perkin Elmer, Watertetten, Germany). PCR conditions were as follows: 1 cycle at 95°C for 5 minutes, 55°C for 5 minutes, and 72°C for 5 minutes. This was followed by 35 cycles of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes, ending with an extension step at 72°C for 10 minutes. From this reaction, 1 \( \mu \text{L} \) was reamplified in the SSP-PCR using primers P13 (5'-CTA GCC GCT CAC CAC TCC TC) and P5G (5'-GAA AAT CCC AGA AAT TTT TCC G) or P4A (5'-GAA AAT CCC AGA AAT TTT TCC A). The allele-specific bases are in bold type (Fig 2).

**Fig 1.** Schematic illustration of the localization of the primers for the nested SSP-PCR and representative results of \( \text{FcRRIa} \) genotype of three individuals: homozygous \( \text{FcRRIa-R/H131} \) (R/H), heterozygous \( \text{FcRRIa-R/H131} \) (R/H), and homozygous \( \text{FcRRIa-H/H131} \) (H/H). For the \( \text{FcRRIa} \)-specific amplification, primers P63 and P52 were used.\(^27\) For the allele-specific amplification, primers P5G (for the \( \text{FcRRIa-R131} \) allele) and P4A (for the \( \text{FcRRIa-R131} \) allele) were combined with a common antisense primer, P13. The 440-bp amplification product of CRP was used as an internal control. The 278-bp fragment represents the allele-specific amplification product from the SSP-PCR of \( \text{FcRRIa} \). Amplification with P13 and P5G (lane 1) and with P13 and P4A (lane 2) shows a homozygous \( \text{FcRRIa-R/H131} \) individual. Amplification with P13 and P5G (lane 3) and with P13 and P4A (lane 4) shows a heterozygous \( \text{FcRRIa-R/H131} \) individual. Amplification with P13 and P5G (lane 5) and with P13 and P4A (lane 6) shows a homozygous \( \text{FcRRIa-H/H131} \) individual. Lane 7 contains a molecular weight standard (100 bp).

**Nucleotide sequencing.** To analyze the polymorphic region, PCR products amplified by primers P63 and P52 were purified by Gene Clean (Dia nova, Hamburg, Germany) and sequenced directly with primer P63 using Sequenase 2.0, as recommended by the manufacturer (Amer sham, Braunschweig, Germany).

**Method validation.** To assess intra-assay reproducibility, EDTA-anticoagulated blood was obtained from 80 patients at two different time points. In a blinded manner, these 160 samples were typed for \( \text{FcRRIa} \) polymorphism. To validate the inter-assay reproducibility, leukocytes from genotyped donors were phenotyped for the \( \text{FcRRIa} \) polymorphism using monoclonal antibodies (MoAbs) in flow cytometric analysis (fluorescence-activated cell sorting [FACS]; n = 340) and \( \text{H}-\text{thymidine} \) incorporation in a T-cell proliferation assay (n = 272).

**Phenotypic analysis of \( \text{FcRRIa} \) allotypes.** FACS scan analysis was performed according to standard methodology, using MoAb IV.3 (mIgG2b; Medarex, Annadale, NJ) that recognizes monomorphic epitopes on \( \text{FcRRIa} \) and MoAb 41H16 (mIgG2a; kindly provided by Dr B.M. Longenecker, University of Alberta, Edmonton, Alberta, Canada) that reacts preferentially with \( \text{FcRRIa-R131} \).\(^{20}\) The anti-CD3-induced T-cell mitogenesis assays were performed as described by Tax et al.\(^{17}\)
including an extra reaction with human IgG2 anti-CD3 to allow discrimination between FcγRIIa-R/H131 and FcγRIIa-R/R131.20

Statistics. Relative frequencies of the FcγRIIa genotypes were compared using χ² statistics for contingency tables with 2 × 3 fields.31 P values were calculated with the SPSS PC+ statistical package (SPSS Inc, Chicago, IL).

RESULTS

Nested SSP-PCR. Results of the SSP analyses for three representative individuals are shown in Fig 1. Amplification of genomic DNA from donor 1 resulted in a 278-bp specific product with primer P5G, but not with primer P4A. In contrast, with DNA from donor 3, this product could be amplified only with primer P4A. Both primers, P5G and P4A, amplified the 278-bp product from donor 2. In all reactions, the 440-bp internal control fragment of the CRP gene was present. These results indicate that donors 1, 2, and 3 represent homozygous FcγRIIa-R/H131, heterozygous FcγRIIa-R/H131, and homozygous FcγRIIa-R/H131, respectively.

Method validation. Results of the nested SSP-PCR were validated by direct sequencing of the polymorphic region; no discrepancies were found (data not shown). Double sample processing (n = 80), FACS analysis using MoAbs recognizing FcγRIIa-R/H131 and FcγRIIa-R/R131 (n = 340), and anti-CD3-induced T-cell mitogenesis (n = 272) demonstrated the intra- and inter-assay reproducibility to be 100%.

Genotype distribution. The genotype distributions and allele frequencies of the FcγRIIa-R-H131 polymorphism are presented in Table 1. There were no significant differences in the genotype distribution between the two control groups (ie, non-HIT patients with thrombocytopenia or thrombosis and healthy blood donors; P = .45). However, in HIT patients, the FcγRIIa-R/R131 genotype was overrepresented and the FcγRIIa-H/H131 genotype was underrepresented when compared with non-HIT control patients (P < .001) and with healthy blood donors (P = .024). Approximately 50% of subjects in all three groups were FcγRIIa-R/H131 heterozygous.

Correlation between FcγRIIa-R-H131 genotypes and manifestation of TECS. In the subanalysis of 122 well-characterized patients, 68 patients (56%) developed TECS during heparin administration and 54 patients (44%) presented with isolated thrombocytopenia. In HIT patients who developed a TEC during heparin treatment, the FcγRIIa-R/R131 genotype was overrepresented and the FcγRIIa-R/H131 and FcγRIIa-H/H131 genotypes were underrepresented compared with HIT patients presenting with thrombocytopenia only; (P = .036; Table 2).

DISCUSSION

HIT antibodies are known to interact with platelets via the FcγRIIa. The FcγRIIa carries a polymorphic site at position 131 (R-H) that affects its capacity to interact with immune complexes. In our investigation, the FcγRIIa-R/R131 genotype was overrepresented and FcγRIIa-H/H131 was underrepresented in HIT patients compared with non-HIT patients and healthy blood donors. Furthermore, a subanalysis with well-characterized HIT patients indicated a correlation between genotype and clinical manifestations of HIT. We found a significantly higher frequency of FcγRIIa-R/R131 in HIT patients who developed TECS than in patients presenting with isolated thrombocytopenia (P = .036; Table 2).

Four previous studies of the relationship between the FcγRIIa-R-H131 polymorphism and the development of HIT have been reported (Table 3).21-24 In three of these studies, FcγRIIa-R/H131 was found to be overrepresented in HIT patients.21,22,24 In the remaining study, no differences in the distribution of FcγRIIa-R-H131 genotypes were detected.23 It may be that the discrepancy between FcγRIIa genotype distribution in the present study and that of earlier studies is due to the inclusion of a different proportion of HIT patients with TECS. In three of the earlier studies, no data are given regarding the percentage of patients who developed HIT-related thrombocytopenia or HIT-related TECS.21,22,24 However, in the remaining study,23 23 of 36 patients had TECS and no significant differences in the FcγRIIa
genotype distribution were found. The disparity in patient sample sizes between our study and earlier studies might also contribute to differences in results; ie, our study includes many HIT patients relative to the small number of HIT patients included in previous trials.

It is possible that the differing distributions of FcγRIIa allotypes in HIT patients reflect normal variations in the FcγRIIa gene frequency, which are found not only among populations of different ethnic origins,27,32 but also within the Caucasian population, where the gene distribution ranges from 18% to 32% for FcγRIIa-R/H131 allotype, the RRIa-R/R131 allotype, the RRIa-H/H131 allotype, whereas, in patients with the FcγRIIa-H/H131 allotype, the uptake of antibody-coated platelets cannot show interactions among cells of the reticulo-endothelial system, cells from the immune system, and platelets and cannot demonstrate the capacity to remove platelets and immune complexes from the circulation.

In two of the previous studies, the IgG subclass of the HIT antibodies was assessed.23,24 Both studies reported that the FcγRIIa polymorphism is primarily responsible for binding differences in antibodies of the IgG2 and IgG3 subclasses;20 however, this polymorphism also seems to influence the interaction with immune complexes of the IgG1 subclass.24

Like the present study, all four of the earlier studies used functional assays to determine the presence or absence of HIT antibodies. We chose this diagnostic technique because functional tests are based on the activation of platelets via the FcγRIIa receptor, and because we were studying FcγRIIa polymorphism, only patients with antibodies that reacted with isolated platelets cannot show interactions among cells of the reticulo-endothelial system, cells from the immune system, and platelets and cannot demonstrate the capacity to remove platelets and immune complexes from the circulation.

Because individuals with FcγRIIa-H/H131 effectively clear immune complexes, this allotype might be regarded as a protective factor against HIT-related thrombosis. Although there is evidence that platelets expressing the FcγRIIa-R/R131 phenotype interact more strongly with HIT antibodies in vitro than platelets with the FcγRIIa-H/H131 phenotype do,25 this need not contradict our hypothesis. Functional in vitro assays with isolated platelets cannot show interactions among cells of the reticulo-endothelial system, cells from the immune system, and platelets and cannot demonstrate the capacity to remove platelets and immune complexes from the circulation. Because the HIPA test is a functional assay that has been shown to have similar sensitivity and specificity as compared with the 14C-serotonin release assay,26 we do not see a major discrepancy to the laboratory methods reported in previous studies on FcγRIIa-R/H131 polymorphism in HIT patients. However, it is possible that HIT patients not identified in this study might have been identified using a PF4/heparin enzyme-linked immunosorbent assay (ELISA).

Together, our findings and those of other published studies suggest that, although the polymorphism may not be the major risk factor for clinical manifestation of HIT, once HIT develops, patients with the FcγRIIa-R/R131 genotype might be at a higher risk of developing new TECs.

ACKNOWLEDGMENT

The technical assistance of C. Blumentritt and A. Raether is highly appreciated, and the pre-PCR work by Dr K. Olbrich is gratefully acknowledged. We thank S. Owens for editorial help with the language.

Table 3. Summary of Previously Published Studies on the Impact of FcγRIIa-R-H131 Polymorphism in HIT Patients

<table>
<thead>
<tr>
<th>Study</th>
<th>Inclusion Criteria</th>
<th>n</th>
<th>FcγRIIa Genotype*</th>
<th>Control Group</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burgess et al22</td>
<td>Clinical criteria of HIT†</td>
<td>19</td>
<td>0</td>
<td>22</td>
<td>7</td>
</tr>
<tr>
<td>Brandt et al22</td>
<td>Thrombocytopenia or thrombost</td>
<td>96</td>
<td>22 (23)</td>
<td>41 (43)</td>
<td>33 (34)</td>
</tr>
<tr>
<td>Arepally et al23</td>
<td>Clinical criteria of HIT§</td>
<td>36</td>
<td>9 (25)</td>
<td>19 (53)</td>
<td>8 (22)</td>
</tr>
<tr>
<td>Denomme et al24</td>
<td>Clinical criteria of HIT</td>
<td>84</td>
<td>(23)</td>
<td>(41)</td>
<td>(36)</td>
</tr>
</tbody>
</table>

*Values are the number of patients with the percentage in parentheses.
†Thrombocytopenia occurring during heparin administration (resolving after heparin withdrawal), heparin-dependent antibodies detected in patient sera/plasma (platelet aggregometry or 14C-serotonin release assay), and exclusion of other recognized causes of thrombocytopenia.
‡Occurrence of thrombocytopenia or thrombosis during heparin administration and a positive platelet aggregation assay.
§Published clinical criteria26a and diagnosis confirmed by detection of heparin-dependent antibodies (14C-serotonin release assay).
¶Only percentages are given in the publication.
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