Intercellular Adhesion Molecule 1 Gene Polymorphisms in Graves' Disease

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It was recently suggested that genetic factors could play a major role in the development of Graves' disease (GD). The aim of the present study was to evaluate the frequency of the c.721G→A polymorphism and the c.1405A→G polymorphism of the intercellular adhesion molecule 1 (ICAM-1) gene in subjects with GD compared with that in healthy controls, because ICAM-1 was found to play a key role in lymphocyte infiltration into the thyroid gland and the concentration of the soluble form of ICAM-1 correlates significantly with the clinical activity and treatment status in GD. We have analyzed the association of ICAM-1 polymorphisms with the age at onset of GD and the presence of ophthalmopathy. In a group of 235 patients with GD and 211 healthy controls we have shown that polymorphism at position c.721G→A is associated with an earlier age of GD onset and that the c.1405A→G polymorphism of the ICAM-1 gene could predispose to Graves' ophthalmopathy. This suggests that G241R and K469E amino acid substitutions in the ICAM-1 molecule could influence the intensity/duration of the autoimmunity process and the infiltration of orbital tissues. It could be speculated that therapy that modulates ICAM-1 function may delay the onset and/or prolong the remission and/or have an influence on clinical manifestations of GD.

G RAVES’ DISEASE (GD) is believed to be an autoimmune lymphocyte-mediated disease, and both environmental and genetic factors play a role in its pathogenesis (1, 2). It is now clear that hyperthyroidism in GD is caused by thyroid-stimulating antibodies activating the TSH receptor, but the mechanisms of other components of the disease (ophthalmopathy, dermopathy, or myxoedema) and the reasons for clinical heterogeneity are still not well known (1).

It was recently suggested that genetic factors could play a major role in the development of GD (3, 4). The concordance rate for clinically overt disease for monozygotic twins has been reported to be between 22 and 60% in different studies, and the model-fitting analysis of data from the pooled twin Danish cohorts showed recently that 79% of the liability to the development of GD is attributable to genetic factors (3, 4).

There is well-established evidence about the associations of human leukocyte antigen type II class alleles (DR3 and DQA1*0501) and CTLA-4 gene polymorphisms with GD in Caucasians (2, 5-7). Moreover, genome-wide search has identified other susceptibility loci to GD on chromosomes 14q31, 18q21, 20q11, and Xq21 (8, 9), although the ability to detect genes of modest effect on GD development is limited by classical linkage analysis (10).

In the present study we evaluated the association of GD with two recently described single nucleotide polymorphisms of the intercellular adhesion molecule 1 (ICAM-1) gene, which have been suggested to be associated with other autoimmune diseases (11-13). Polymorphism G→A at position c.721 in exon 4 of the ICAM-1 gene results in a change from glycine (GGG) to arginine (AGG) at position 241 in the amino acid chain (G241R), but substitution c.1405 A→G in exon 6 results in a change from lysine (AAG) to glutamic acid (GAG) at position 469 in the amino acid chain (K469E).

ICAM-1 is a surface glycoprotein, expressed on vascular endothelium, macrophages, and activated lymphocytes, that belongs to the Ig superfamily and functions as a receptor for leukocyte function-associated antigen 1 (LFA-1) and macrophage differentiation antigen 1 (14, 15). ICAM-1 plays a key role in the process of leukocyte circulation and extravasation from the blood into the areas of inflammation and delivery of costimulatory signals of immunocompetent cells (14, 16).

The ICAM-1 molecule is expressed on activated T, B, dendritic, and endothelial cells in the thyroid of GD patients, and in functional and experimental studies the ICAM-1/LFA-1 pathway was found to play a major role in leukocyte infiltration into thyroid, the development of an autoimmune response, and thyrocyte proliferation in GD (16-18). There is, however, some controversy as to whether the ICAM-1 molecule is expressed on the thyroid follicular cells derived from patients with GD. In contrast to the studies by Fukazawa et al. (19) and Arreaa et al. (20), who found a high expression of ICAM-1 molecule on thyroid epithelial cells using immunohistochemistry and/or flow cytometry methods, other researchers have failed to detect the ICAM-1 antigen in vivo on thyroid cells in GD (21, 22). On the contrary, it is well documented that patients with untreated GD have a high serum level of a soluble form of ICAM-1 (sICAM-1), and the concentrations of this adhesion molecule correlate with the activity of the disease, probably reflecting an ongoing immune process (23-27).

We have, therefore, examined the frequency of the known polymorphisms of the ICAM1 gene: the c.721G→A poly-
morphism in exon 6 and the c.1405A→G polymorphism in exon 6 in subjects with GD compared with that in healthy controls. Moreover, we have evaluated the differences between the frequencies of ICAM-1 gene alleles in patients with GD onset before 40 yr of age (early onset) compared with those in subjects with a later onset of disease. The association between age at onset and different human leukocyte antigen genotypes has been previously reported in patients with GD (28, 29). In addition, distinct ICAM-1 gene allelic associations according to age at onset have been observed in other autoimmune diseases (11). The criteria we used to stratify the patients into those with early and later ages of onset of disease were based on the epidemiological observations of the highest risk of the onset of GD between the ages of 40 and 60 yr (1).

Finally, we examined the association between the studied polymorphisms and Graves’ ophthalmopathy (GO), because a strong immunoreactivity for ICAM-1 was detected in the blood vessels, perimysial fibroblasts surrounding the extracellular muscle fibers, and connective tissue in the retroocular tissues of patients with GO (30). Moreover, serum levels of sICAM-1 are even higher in subjects with GO than in patients without ophthalmopathy and correlate with the severity of eye disease and the treatment/clinical status (23, 31, 32).

Subjects and Methods

Subjects

The study was carried out in 235 unrelated Polish origin Caucasian patients (female/male ratio, 5:7:1) with GD: 136 subjects with disease onset under 40 yr of age (range, 12–39 yr; mean, 27.6 ± 7.5 yr) and 99 subjects with the onset at 40 yr of age or older (40–77 yr; mean, 50.4 ± 8.8 yr). Patients with GD were sequentially recruited from the endocrinology outpatient clinic from the Białystok region of Poland between April 2001 and November 2002. GD was diagnosed on the basis of clinical observation (diffuse goiter), biochemical criteria of thyrotoxicosis (TSH < 0.05 and increased free T3 and/or free T4), and the presence of TSH receptor antibodies (TSHR Ab; >2 IU/μl) (33). However, not all patients with GD have positive TSHR Ab (using the newly developed recombinant TSH binding-inhibiting Ig assay, TSH receptor antibodies are detected in ~95% of patients with active GD); therefore, we decided to include in our study only the patients with positive TSHR Ab and avoid an admixture of disseminated thyroid autonomy (34, 35).

Thyroid eye disease was diagnosed by experienced ophthalmologists and was classified using the NOSPECS classification (36). For the statistical analysis, patients with classes 2–6 were considered as having GO.

Two hundred and eleven healthy Caucasian volunteers (female/male ratio, 5:1:1) from the same region of Poland with no family history of GD or other autoimmune diseases served as the control group. Informed consent was obtained from the GD patients and healthy controls, and the study was approved by the ethics committee of the Medical University of Białystok. TSHR Ab were measured by RIA using TRAK kit (BRAHMS, Berlin, Germany) (34).

Genotype analysis

DNA was extracted from the peripheral blood leukocytes, and polymorphisms at positions c.721 (exon 4) and c.1405 (exon 6) in the ICAM-1 gene were detected by the PCR sequence-specific primers method (37).

For position c.721 in exon 4, two sequence-specific forward primers [5′-GTGTTCTGGTTCCTGAGC-3′ (allele c.721 G) and 5′-GTGTTCTGTCCCTTGAGC-3′ (allele c.721 A)] and for position c.1405 (exon 6) two sequence-specific reverse primers [5′-GCACATTACGGTCAC-CTC-3′ (allele c.1405G) and 5′-GCACATTACGGTCACCTT-3′ (allele c.1405A)] were used.

For the control PCRs, 5′-ATGATGTTGACCTTTCCAGGG-3′ and 5′-TTCGTTAATTTTTGACCTGGTC-3′ primers were used for amplification of the 256-bp fragment from exon 15 of the adenomatous polyposis coli gene.

Each of the four primer mixes contained a combination of one forward primer and one reverse primer for the sequence-specific amplifications and both control primers. Reactions were carried out in a PTC-200 thermal cycler (MJ Research, Cambridge, MA) under the following conditions: 2 min at 96 C; 6 cycles of 30 sec at 96 C, 45 sec at 69 C, and 45 sec at 72 C; 21 cycles of 30 sec at 94 C, 45 sec at 65 C, and 45 sec at 72 C; and 5 cycles of 30 sec at 96 C, 60 sec at 55 C, and 120 sec at 72 C.

The genotypes were determined according to the presence of the specific PCR products of expected length (927 bp) in the presence of the control product after 2% agarose gel electrophoresis, followed by ethidium bromide staining and UV visualization.

To confirm the results of the PCR sequence-specific primers method, appropriate fragments of exons 4 and 6 of the ICAM-1 gene from 50 randomly selected subjects from both of the studied groups were amplified and sequenced on ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA) with the following pairs of primers: for exon 4, 5′-GGAAACCATGCTCCGGG-3′ and 5′-GGTGGTTATGGCATTAGTG-3′; and for exon 6, 5′-GATTGAAAGACCCAGCAAG-3′ and 5′-GATGCTGCTGATGCTGACTG-3′. The results of both methods were identical.

The nomenclature for polymorphism description was adopted from Nomenclature Working Group (http://archive.uwcm.ac.uk/uwcm/mg/docs/mut_nom.html).

Statistical analysis

The differences in the distribution of alleles and genotypes between the studied groups were estimated by χ2 test for 2 × 2 or 2 × 3 tables (SAS/STAT 6.12, Cary, NC; and Statistica 5.5, StatSoft, Tulsa, OK). Statistical significance was defined as P < 0.05. When necessary, the P value was corrected for the number of variables tested [corrected P (Pc) = P × 14].

Results

Genotypes and allele frequencies of c.721G→A ICAM-1 gene polymorphism

The frequencies of alleles and the distribution of genotypes of the ICAM-1 gene polymorphism in GD patients and control subjects are presented in Tables 1 and 2, respectively. At position c.721 of the ICAM-1 gene we observed a higher frequency of allele A in patients with GD compared with the healthy controls, but the difference did not reach statistical significance after P value correction for multiple comparison (χ2 = 4.76; P = 0.029; Pc = 0.406).

After age at onset stratification, we observed that patients with an earlier (<40 yr of age), but not with a later (≥40 yr of age) onset of the disease had higher frequencies of allele A compared with healthy controls (χ2 = 9.22; P = 0.0024; Pc = 0.034).

The differences in genotype distribution between patients with the earlier onset of GD and the control group reflected an increase in c.721GA genotypes and a decrease in c.721GG genotypes (χ2 = 12.54; P = 0.002; Pc = 0.028; Table 2).

Genotype and allele frequencies of c.1405A→G ICAM-1 gene polymorphism

At position c.1405 of the ICAM-1 gene there were no statistically significant differences in alleles and genotype frequencies between the GD patients and controls (Table 2). However, we observed a higher frequency of allele G at position c.1405 in GD patients with a clinical diagnosis of ophthalmopathy compared with subjects without thyroid...
TABLE 1. Frequency of alleles of ICAM-1 gene polymorphisms in GD and controls

<table>
<thead>
<tr>
<th>Locus c.721</th>
<th>GD onset</th>
<th>GD ophthalmopathy</th>
<th>All GD</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;40 yr</td>
<td>≥40 yr</td>
<td>With</td>
<td>Without</td>
</tr>
<tr>
<td></td>
<td>(n=136)</td>
<td>(n=99)</td>
<td>(n=108)</td>
<td>(n=127)</td>
</tr>
</tbody>
</table>
| G           | 189 (69.5)
| A           | 83 (30.5)        | 156 (78.8) | 155 (71.8) | 190 (74.8) | 345* (73.4) | 336 (79.6) | 36 (80.4) |
|            |          |                   | 61 (28.2) | 64 (25.2)  | 125 (26.6) | 86 (20.4)  |            |            |
| Locus c.1405|          |                   |        |           |            |            |            |            |
| G           | 121 (44.5) | 81 (40.9) | 109 (50.5)
| A           | 151 (55.5) | 117 (59.1) | 107 (49.5) | 161 (36.3) | 268 (57.0) | 261 (61.8) | 14 (34.1) |

Values given are the number of alleles, with the percentage in parentheses.

a The difference in the frequencies of alleles between all GD patients and controls was not statistically significant after P value correction ($\chi^2 = 4.76; P = 0.029; P_c = 0.406$).

b The distribution of allele frequencies differed significantly between the subjects with onset of GD at less than 40 yr and compared with subjects with a later onset was not statistically significant after $P$ value correction ($\chi^2 = 5.08; P = 0.024; P_c = 0.336$).

c The difference in the frequencies of alleles between subjects with the onset of GD at less than 40 yr compared with subjects with a later onset was not statistically significant after $P$ value correction ($\chi^2 = 5.08; P = 0.024; P_c = 0.336$).

d The difference in the frequencies of alleles between subjects with the onset of GD at less than 40 yr compared with subjects with a later onset was not statistically significant after $P$ value correction ($\chi^2 = 5.08; P = 0.024; P_c = 0.336$).

e The distribution of allele frequencies differed significantly between GD patients with ophthalmopathy and controls ($\chi^2 = 8.87; P = 0.0029; P_c = 0.041$).

f The distribution of allele frequencies differed significantly between GD patients with or without ophthalmopathy ($\chi^2 = 9.14; P = 0.0025; P_c = 0.035$).

TABLE 2. Frequency of genotypes of ICAM-1 gene polymorphisms in GD and controls

<table>
<thead>
<tr>
<th>Locus c.721</th>
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<th>GD ophthalmopathy</th>
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<td></td>
<td>(n=136)</td>
<td>(n=99)</td>
<td>(n=108)</td>
<td>(n=127)</td>
</tr>
</tbody>
</table>
| GG          | 61 (44.8)
| GA          | 67 (49.3) | 38 (38.4) | 52 (48.1) | 68 (53.5) | 120 (51.0)
| AA          | 8 (5.9)   | 2 (2.0)            | 5 (4.6) | 5 (3.9)  | 10 (4.3)  | 10 (4.7)  | 135 (64.0) |
|            |          |                   |        |           |            |            |            |            |
| Locus c.1405|          |                   |        |           |            |            |            |            |
| GG          | 28 (20.6) | 19 (19.2) | 30 (27.8)
| GA          | 65 (47.8) | 43 (43.3) | 45 (41.7) | 63 (49.6) | 108 (46.0) | 93 (44.1) | 84 (39.8) |
| AA          | 43 (31.6) | 37 (37.4) | 33 (30.6) | 47 (37.0) | 80 (34.0)  |            |            |            |

Values given are the number of subjects, with the percentage in parentheses.

a The difference in the frequencies of genotypes between all GD patients and controls was not statistically significant after $P$ value correction ($\chi^2 = 8.51; P = 0.014; P_c = 0.196$).

b The distribution of genotype frequencies differed significantly between subjects with GD onset at less than 40 yr and controls ($\chi^2 = 12.54; P = 0.002; P_c = 0.028$).

c The difference in the frequencies of genotypes between patients with ophthalmopathy and the controls was not statistically significant after $P$ value correction ($\chi^2 = 5.08; P = 0.024; P_c = 0.224$).

d The difference in the frequencies of genotypes between GD patients with or without ophthalmopathy was not statistically significant after $P$ value correction ($\chi^2 = 7.84; P = 0.02; P_c = 0.28$).

Other immune response alterations in humans (12, 37). It has been shown that polymorphisms in the ICAM-1 gene can modulate immune function in several autoimmune diseases, such as multiple sclerosis, type 1 diabetes, or Crohn’s disease (11, 38, 39). A higher frequency of allele A (R241) and genotype c.721GA (241RG) and an association with the clinical manifestation (antibodies status) have been found in patients with ulcerative colitis (13). Moreover, similar to our observations, Nishimura et al. (11) found that a polymorphism in exon 6 of the ICAM-1 gene affects the age at onset of type 1 diabetes. Allele A at position c.721 of the ICAM-1 gene was observed twice as frequently in patients with renal allograft failure as in long-term survivors after renal transplantation (12). The more rapid renal graft failure was associated with the E469 ICAM-1 variant in the recipient (12).

We believe that our present observations are in agreement with previous studies concerning the role of ICAM-1 in the pathogenesis of GD. It has been shown in in vitro experiments that ICAM-1 plays an important role in the process of lym-
phocyte attachment to cultured Graves’ thyroid cells (17). Inflammatory cells from thyroids of patients with GD express a high quantity of ICAM-1, and thyroid cell proliferation activated by infiltrating lymphocytes could be blocked by ICAM-1 and/or LFA-1 monoclonal antibodies (17, 40). Moreover, anti-TSHR Ab from subjects with GD increase the expression of ICAM-1 on the surface of human thyroid cells, and this mechanism has been suggested to play a main role in promoting lymphocyte recruitment to the thyroid gland (41).

It has been previously suggested that the polymorphism in the ICAM-1-binding site (exon 4) and in the fifth Ig-like domain (exon 6) changes ICAM-1 properties: cell adhesion and the activity of costimulation. Amino acid substitutions in domain III of ICAM-1 were shown to alter binding properties to macrophage differentiation antigen 1 molecule in vitro studies (14). It is highly probable that a polymorphism in this region can influence ICAM-1 function in vivo. We can only speculate that these changes in ICAM-1 structure and function could influence the clinical manifestations of GD. It is well known that gene polymorphisms in other molecules expressed on immunocompetent cells (human leukocyte antigen and cytotoxic T lymphocyte-associated antigen 4) could influence the susceptibility to GD and/or the age of onset of the disease (2, 5, 28).

Patients with active GD have elevated serum concentrations of sICAM-1, which correlate with the activity of the disease, reflecting the ongoing immune processes (23, 25, 31, 42). However, the physiological role of sICAM-1 is not well defined; some studies have suggested that by competition with membrane-binding ICAM-1, the soluble form could regulate the activity of the immune system (43). Rothlein et al. (43) reported that the circulating form of ICAM-1 contains all five extracellular domains of membrane ICAM-1 as well as the ability to bind specifically to LFA-1. It is possible that the studied polymorphisms of the ICAM-1 gene, by changing the structure of the soluble form of ICAM-1, could influence sICAM-1 function and by that mechanism have an impact on the onset of the clinical symptoms and signs of GO.

It is important to mention that the mechanism of inflammatory cell infiltration into the thyroid gland in GD could also comprise the involvement of adhesion molecules other than ICAM-1. Marazuela et al. (44) suggested the important role of ICAM-3 in thyroid autoimmunity, as they observed that most of the intrathyroidal mononuclear cells expressed the ICAM-3 adhesion molecule in patients with GD. However, the ICAM-3 protein structure is 48% identical with ICAM-1; its expression is regulated by another gene (45). No association between the levels of sICAM-1 and sICAM-3 was observed in the sera of patients with GD or other autoimmune diseases (46).

In summary, our study shows that ICAM-1 gene polymorphisms are associated with GD and may influence the age of the onset of GD. It may be suggested that amino acid substitution (G241R and K469E) caused by the observed polymorphisms of the ICAM-1 gene could influence the intensity/duration of the autoimmune process and the infiltration of extrathyroidal (orbital) tissues, probably as a result of altered interaction between Ig-like domains of ICAM-1 and respective ligands. As it was shown in animal model that transient blockade of the LFA-1/ICAM-1 pathway by adenovirus gene therapy protects autoimmunity development without causing general immunosuppression (16), one could speculate that therapy that modulates ICAM-1 function may delay the onset, prolong the remission, and/or have an influence on the clinical manifestations of GD.

Acknowledgments

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References

6. Maciel LMZ, Rodrigues SS, Dibbern RS, Navarro PAA, Donadi EA 2001 Association of the HLA-DRB1*0101 and HLA-DQA1*0501 alleles with Graves’ disease in a population representing the gene contribution from several ethnic backgrounds. Thyroid 11:31–35
and ICAM-1 (CD54) molecule on the surface of thyroid cells from patients with Graves’ disease. Thyroid 3:285–289