Molecular Human Reproduction Vol.7, No.12 pp. 1167–1172, 2001

HLA-G genotypes and pregnancy outcome in couples with unexplained recurrent miscarriage

C.L.Aldrich[1], M.D.Stephenson[2], T.Karrison[3], R.R.Odem[4], D.W.Branch[5], J.R.Scott[5], J.R.Schreiber[4] and C.Ober[1,6]

Departments of [1]Human Genetics and [3]Health Studies, The University of Chicago, [2]Department of Obstetrics and Gynecology, University of British Columbia, [4]Department of Obstetrics and Gynecology, Washington University School of Medicine and [5]Department of Obstetrics and Gynecology, University of Utah School of Medicine, USA

[6]To whom correspondence should be addressed at: Department of Human Genetics, The University of Chicago, 920 E. 58th Street, Rm. 507C, Chicago, IL 60637, USA. E-mail: c-ober@genetics.uchicago.edu

***HLA-G* is a non-classical human leukocyte antigen expressed primarily in fetal tissues at the maternal–fetal interface. This expression pattern is unique among *HLA* genes and suggests that *HLA-G* may be involved in interactions that are critical in establishing and/or maintaining pregnancy. To evaluate the role of polymorphisms at this locus in maternal–fetal interactions, 113 couples with unexplained recurrent miscarriage were genotyped for seven polymorphisms that define 12 *HLA-G* alleles. Logistic regression analysis was used to assess whether *HLA-G* genotypes were associated with an increased risk for a subsequent miscarriage. The presence of an HLA-G*0104 or HLA-G*0105N allele in either partner was significantly associated with an increased risk for miscarriage, after adjustment for maternal age, number of previous miscarriages, history of a previous liveborn, and treatment with paternal mononuclear cells. The *0104 and *0105N alleles are defined by polymorphisms in the α-2 domain and encode protein variants that are present only in the full-length HLA-G1 protein. The significant genotype-specific risk in this population suggests that allelic variation in the α-2 domain of the HLA-G1 isoforms contributes to recurrent miscarriage.**

Key words: human leukocyte antigen/recurrent miscarriage/HLA-G

Introduction

The first reports of a non-classical *HLA* gene expressed in the extravillous cytotrophoblast of the placenta (Redman *et al.*, 1984; Ellis *et al.*, 1986) and subsequently identified as *HLA-G* (Ellis *et al.*, 1990; Kovats *et al.*, 1990), brought the promise of new insight into the 'riddle of the fetal allograft' (Hunt *et al.*, 1992; Schmidt and Orr, 1993). However, as data about this gene continue to accumulate, a clear idea of its function in maternal–fetal interactions remains elusive. This is due, in part, to the inherent complexities of HLA-G biology including its pattern of differential RNA splicing, the lack of antibodies to distinguish among all HLA-G protein isoforms, the co-expression of HLA-E and HLA-C in the extravillous cytotrophoblast, and the absence of a good animal model. Although splice variants of HLA-G were described as early as 1992 (Ishitani and Geraghty, 1992), to date there is little known about the differential roles of these proteins in pregnancy. Ribonuclease protection data suggest that the full-length membrane-bound variants (HLA-G1) account for the most abundant message in placental tissue (Ishitani and Geraghty, 1992; Hiby *et al.*, 1999), and may be the only isoform expressed at the cell surface (Bainbridge *et al.*, 2000a; Mallet *et al.*, 2000). On the other hand, soluble forms of the G1 isoform (Rebmann *et al.*, 2001) as well as the shorter (HLA-G2) isoform have been detected in maternal serum (Hunt *et al.*, 2000). However, the lack of antibodies that discriminate between the protein isoforms has made it difficult to characterize the natural history and differential expression pattern of these proteins. Furthermore, while HLA-G is the predominant HLA protein in the extravillous cytotrophoblast (Hunt and Orr, 1992; Le Bouteiller and Blaschitz, 1999), HLA-C and HLA-E are co-expressed (Wei and Orr, 1990; Houlihan *et al.*, 1995; King *et al.*, 1996), potentially confounding functional studies. Finally, the use of a model organism, such as mouse with its well-characterized histocompatibility genes and powerful genetic toolbox, is hampered by the differences in placental development and anatomy (Wooding and Flint, 1994) and the lack of a murine HLA-G homologue (Cao *et al.*, 1999).

An alternative approach to elucidating the role of HLA-G in pregnancy is to study the relationship between *HLA-G* genotypes and expression patterns in patients with abnormal

pregnancies. For example, a number of studies of HLA-G in pre-eclamptic (PE) pregnancies have suggested that expression of HLA-G protein is reduced in term placentas of PE compared with normal pregnancies (Hara *et al.*, 1996; Lim *et al.*, 1997). However, the lack of association between a null allele (HLA-G*0105N) for the full length isoform and PE suggests that the G1 isoform is not likely to be involved (Aldrich *et al.*, 2000).

Surprisingly, there have been relatively few studies of HLA-G and miscarriage, the most common disorder in pregnancy. Three small studies examined a subset of *HLA-G* polymorphisms (Karhukorpi *et al.*, 1997; Penzes *et al.*, 1999; Yamashita *et al.*, 1999), each in fewer than 40 couples with recurrent miscarriage. None of these studies found an association between *HLA-G* genotypes and recurrent miscarriage, but the small sample sizes and the incomplete genetic analyses make it difficult to draw conclusions from these studies. It is particularly noteworthy that these studies did not genotype their subjects for the *HLA-G**0104 or *HLA-G**0105N alleles. Recently, Pfeiffer and colleagues studied all *HLA-G* alleles in 78 couples with recurrent miscarriage and 52 controls. They reported an increased frequency of the HLA-G*01013 and HLA-G*0105N alleles in the recurrent miscarriage cases compared with controls, and suggested that this subgroup of patients might benefit from treatment with immunotherapy, such as paternal cell immunization (Pfeiffer *et al.*, 2001).

We initiated this study to fully characterize the *HLA-G* alleles in both partners of 113 couples with a history of three or more unexplained miscarriages and who participated in a randomized clinical trial evaluating the efficacy of treatment with paternal mononuclear cell immunization (Ober *et al.*, 1999). To determine whether *HLA-G* genotype is a marker for a subgroup of patients who might benefit from immunotherapy, we also examined interactions between *HLA-G* genotype and efficacy of treatment and the effect of genotype on subsequent pregnancy outcome. These studies provide further insight into the possible role of HLA-G in pregnancy.

Materials and methods

Patients

Patients were recruited from six centres in the USA and Canada as part of the Recurrent Miscarriage (REMIS) study, a randomized clinical trial evaluating the efficacy of paternal mononuclear cell immunization as a treatment for unexplained recurrent miscarriage (Ober *et al.*, 1999). All participating centres had approval from the local institutional review board and all patients gave their informed consent. Patients were enrolled in the study if they had three or more previous miscarriages (excluding chromosomally abnormal fetuses and ectopic pregnancies), no more than one live born child with the current partner, were aged <41 years at the time of recruitment, and had no identifiable causes for their previous history of recurrent miscarriages, including anatomic, endocrinologic, cytogenetic and autoimmunologic causes. The diagnostic screening protocol included cytogenetic studies in both parents, luteal-phase serial progesterone measurements or in-phase endometrium biopsy, measurement of thyrotrophin concentrations in serum, assays of antibodies to cardiolipin and lupus anticoagulant, and analysis of intrauterine contour by hysterosalpingography, sonohysterography or hysteroscopy. Patients were randomized to a treatment (immunization with husbands' mononuclear cells) or control (immunization with saline) group; all study subjects received 'tender loving care' in the first trimester, including weekly ultrasound examinations (Ober *et al.*, 1999).

Forty couples who were randomized but did not achieve a pregnancy within the 12 month study window (Ober *et al.*, 1999) were not included in this study because DNA was not collected until pregnancy occurred. Of the 131 couples who achieved a pregnancy in the REMIS trial, 113 were included in this study. Sixteen couples were excluded because DNA was not obtained from both partners. Two additional couples were excluded because each of the partners had different *HLA* alleles, which precluded them being classified into either a high or low risk genotypic class (see Results). Among the 113 couples, 107 were Caucasian, two were African-American, two were Hispanic, one was East Asian and one was Asian-Indian. Mean maternal age was 32.6 years (SD 4.8; range 22–41) and the mean number of previous miscarriages was 4.4 (SD 1.7; range 3–13). Twenty-eight (25%) of the couples had a previous liveborn. A total of 61 received treatment with their partner's mononuclear cells and 52 received saline as a placebo. There were 63 subsequent successful outcomes (i.e. delivered a live born child) and 50 experienced another miscarriage. All losses occurred prior to 28 weeks gestation.

HLA-G genotyping

Twelve alleles at the *HLA-G* locus were genotyped in both partners by probing dot-blotted DNA with sequence-specific oligonucleotide probes. Exons 2 and 3 of *HLA-G* were amplified with primers complementary to intron 1 and 3 sequences (Primer G2i5: GAGGGTCGGGCGGGTCTCAAC and Primer G3i31: CTCCTTGTGCTAGGCCAGGCTGA). The polymerase chain reaction (PCR) included an initial denaturing step at 95°C for 3 min, followed by 35 cycles at 95°C for 15 s, 65°C for 80 s, and 72°C for 30 s with a final extension of 72°C for 5 min. These highly stringent PCR conditions and intronic primers specifically amplified exons 2 and 3 of *HLA-G,* as confirmed by sequencing of representative PCR products. The PCR product was denatured and 'dotted' on nylon membranes by vacuum. A positive and negative control for each polymorphism were included on each blot. The blots were hybridized to single-stranded oligonucleotide probes specific for the nucleotide polymorphisms at codons 31 (A/T, Probe GP31a: XGTGGACGACACGCAGTTCG and Probe GP31t: XGTGGACGACTCGCAGTTCG), 57 (G/A, Probe GP57g: XCCCAATACTCCGGCCCCTC and Probe GP57a: XGAGGGGCCAGAGTATTGGG), 69 (C/T, Probe GP69c: XACGCCAAGGCCCACGCACA and Probe GP69t: XCTGTGCGTGAGCCTTGGCG), 93 (C/T, Probe GP93c: XCAGGTTCTCACACCCTCCAG and Probe GP93t: XCTGGAGGGTATGAGAACCTG), 107 (A/T, Probe GP107a: XGTCCGACGGACGCCTCCT and Probe GP107t: XGTCCGACGGTCGCCTCMT), 110 (C/A, Probe GP110c: XGGCGCCTCCTCCGCGGGT and Probe GP110a: XGGCGCCTCATCCGCGGGT) or 130 (C/ΔC, Probe GP130w: XGAACGAGGACCTGCGCTCC and Probe GP130d: XGAACGAGGACTGCGCTCCT) (X = biotin for all probes). The specific pattern of positive probes was used to determine the following *HLA-G* alleles: *HLA-G**01011/6, *01012/g (van der Ven *et al.*, 1998), *01013, *01014, *01015, *01017, *01018, *0103, *01041, *01043, *01046, (van der Ven *et al.*, 1998), and *0105N (Table I). This protocol identified all polymorphisms that result in amino acid substitutions or that otherwise affect the protein structure, but probes were not designed to distinguish among all silent variants of the *0101 allele (*01011/*01016 and *01012/*0101g) and the *0104 allele (*01041/*01042 and 01042/*01043).

Table I. Polymorphic variants in *HLA-G* investigated in this study. The amino acids with the polymorphic nucleotides are shown along the top row and allele names are shown down the first column. The three polymorphisms at amino acids 31, 110, and 130, that affect the protein sequence, are shown in boldface type. The mutation at amino acid 130 is a deletion of a single cytosine at nucleotide 1597

Allele	Thr31Ser	Pro57	Ala69	His93	Gly107	Leu110Ile	130ΔC	Frequency in women	Frequency in men
*01011/6	A	G	C	C	A	C	C	0.443	0.432
*01012/g	A	A	C	T	A	C	C	0.317	0.361
*01013	A	A	C	C	T	C	C	0.077	0.067
*01014	A	G	T	C	A	C	C	0	0
*01015	A	G	C	C	T	C	C	0.012	0
*01017	A	A	C	T	T	C	C	0.020	0
*01018	A	A	C	C	A	C	C	0.012	0.021
*0103	**T**	G	C	C	A	C	C	0.024	0.029
*01041,2,3	A	A/G	C	C	A	**A**	C	0.073	0.080
*0104b	A	A	C	T	A	**A**	C	0.004	0.004
*0105N	A	A	C	T	A	C	Δ	0.016	0.004

Statistical analysis

The proportion of couples with successful pregnancy outcomes following entry into the clinical trial (Ober *et al.*, 1999) was compared among HLA genotypes by Fisher's exact test for general r×c tables (Mehta and Patel, 1983). Because both maternally- and paternally-inherited *HLA-G* alleles are expressed in placental tissues (Hashimoto *et al.*, 1997; Hiby *et al.*, 1999), both male and female partners' genotypes were considered.

Logistic regression was used to assess the effect of *HLA-G* genotypes on pregnancy outcome. Three epidemiological variables that are known to influence recurrent miscarriage (maternal age, number of previous miscarriages, previous live born child) were included in the analyses, as in our previous report (Ober *et al.*, 1999). In addition, the assigned treatment group in the randomized trial (treatment = 1/control = 0) was included as a fourth covariate. A fifth covariate was a genotype indicator for each couple, categorizing couples for the presence or absence of the 'high risk' genotypes identified in the first analysis, described above. In addition, interactions between the epidemiological/clinical and genetic variables were evaluated.

Results

HLA-G allele frequencies in wives and husbands are shown in Table I. Because the frequency of the *HLA-G**01013 allele did not differ between couples with successful and failed pregnancies (0.27 and 0.26 respectively), we considered this allele together with the other *0101 alleles that do not differ at the amino acid level (Table I). Success and failure rates for the *HLA-G* allelic classes, i.e. those that differ at the amino acid level, are shown in Table II. We separately examined couples in which both partners carried two copies of the common *0101 alleles (including *01011, *01012, *01013, *01014, *01015, *01016, *01017, *01018 and *0101g) and those couples in which at least one partner carried at least one copy of the *0103 (0103-positive), *0104 (0104-positive) or *0105N (0105N-positive) alleles. In two couples with successful outcomes, one partner was positive for *0103 and the other partner was positive for *0104. These couples could not be categorized and were therefore excluded from the analyses. Of those who remained in the study, one male partner was homozygous for the *0104 and another male partner was homozygous for the *0103 allele. All remaining individuals with a non-*0101 allele on one chromosome carried the common *0101 allele on the other chromosome. Success rates differed between couples with different *HLA-G* genotypes ($P = 0.010$) (Table II). The results were similar if couples with karyotypically abnormal conceptuses were excluded ($P = 0.009$). It is notable that four female and one male partner carried the *HLA-G**0105N allele. This is a null allele for the full-length, G1 isoform and is particularly common among populations of African descent but relatively rare among Caucasians (Hviid *et al.*, 1997; Ober *et al.*, 1998; van der Ven *et al.*, 1998; Alvarez *et al.*, 1999; Hiby *et al.*, 1999; Loke *et al.*, 1999). Surprisingly, in this sample four of the five carriers of this null allele were Caucasian. Overall, failure rates were higher in couples where at least one partner carried the *0104 or *0105N alleles. The frequency of *0104 or *0105N carriers was higher in 35 couples with five or more losses compared with 78 couples with only two or three previous losses (37 versus 26%), however this difference did not reach statistical significance ($P = 0.214$). Based on these results and the fact that both the *0104 and *0105N polymorphisms are in the α-2 domain and neither affect the G2 isoforms, couples were classified as *0104- or *0105N-positive versus all others for the logistic regression analysis.

Table II. Number (%) couples with successful or failed pregnancies by genotype group. Couples were categorized as follows: *HLA-G**0101/*0101 genotype in both partners, the presence of *HLA-G**0103 in either partner (*0103-positive), the presence of *HLA-G**0104 in either partner (*0104-positive), or the presence of *HLA-G**0105N in either partner (*0105-positive). See text for additional details. Fisher's exact test (4×2 table): $P = 0.010$

HLA-G genotype	Success	Failure
*0101/*0101	48 (67%)	24 (33%)
*0103-positive	4 (50%)	4 (50%)
*0104-positive	10 (36%)	18 (64%)
*0105N-positive	1 (20%)	4 (80%)
Total	63 (56%)	50 (44%)

Logistic regression analysis was used to adjust for epidemiological and clinical variables and to further quantify the risk associated with *HLA-G* genotypes (Table III). The number of previous miscarriages [odds ratio (OR) 1.36; 95% confidence

Table III. Results of logistic regression analysis of risk factors for pregnancy loss

Independent variables	Odds ratio Exp(β)	95% confidence interval	*P*-value
Number of previous miscarriages	1.36	1.04, 1.770.	024
Maternal age	0.94	0.86, 1.03	0.160
Previous live born	0.47	0.17, 1.31	0.150
Treatment (mononuclear cell immunization)	2.36	1.01, 5.51	0.046
HLA-G genotype (*0104- or *0105N-positive)	3.62	1.45, 9.03	0.006

interval (CI) 1.04, 1.77; $P = 0.024$] and treatment with paternal mononuclear cell immunization (OR 2.36; 95% CI 1.01, 5.51; $P = 0.046$) were associated with increased loss rates, as previously reported (Ober *et al.*, 1999). Neither the absence of a previous liveborn nor maternal age was significantly associated with outcome in this sample. The lack of association with maternal age was surprising because of the well-established association between maternal age and risk of miscarriage (Wilson *et al.*, 1984). However, the couples in this study were selected because of a history of three or more prior miscarriages and the effects of maternal age on miscarriage risk may be less important in these couples, as our data suggest.

The presence of the *0104 or *0105N alleles in either partner was a highly significant predictor of pregnancy outcome (OR 3.62; 95% CI 1.45, 9.03; $P = 0.006$) (Table III). These results were still significant after exclusion of the seven non-Caucasian couples (OR 3.58; 95% CI 1.4, 9.10; $P = 0.008$), patients with a previous liveborn (OR 2.78; 95% CI 1.02, 7.52; $P = 0.045$) and couples with a subsequent karyotypically abnormal miscarriage (OR 4.94; 95% CI 1.83, 13.31; $P = 0.002$). There were no significant interactions between being positive for *0104 or *0105N and any of the demographic or clinical variables, including treatment with mononuclear cell immunization ($P > 0.10$ for all interactions). Because of the previous results of Pfeiffer *et al.* (Pfeiffer *et al.*, 2001), we also separately examined the effects of the *01013 allele in the logistic model. In our patient sample, this allele was not a significant predictor of subsequent pregnancy outcome (OR 1.26; 95% CI 0.51, 3.10; $P = 0.614$).

Discussion

Although it has been known for nearly a decade that HLA-G is the major HLA molecule expressed in the extravillous cytotrophoblast of the placenta, the role of HLA-G with respect to pregnancy outcome remains to be elucidated. This study reports a novel association of the *HLA-G**0104 allele with pregnancy loss in couples with unexplained recurrent miscarriage and further supports an association between the *HLA-G**0105N allele and recurrent miscarriage (Pfeiffer *et al.*, 2001). The association with the *0104 allele was unexpected as the allele is defined by a conservative amino acid change of a leucine to an isoleucine at amino acid 110 in exon 3, which encodes the α-2 domain of the full-length G1 isoforms. These data suggest that there may in fact be a functional difference between these alleles and that the HLA-G1 isoforms play a critical role in fetal survival. The presence of five individuals carrying the *HLA-G**0105N allele was also surprising in this predominantly Caucasian population because this allele is generally rare or absent in populations of European descent (Hviid *et al.*, 1997; Ober *et al.*, 1998; van der Ven *et al.*, 1998; Alvarez *et al.*, 1999; Hiby *et al.*, 1999; Loke *et al.*, 1999), although this frequency is similar to that found in another sample of Caucasian couples with recurrent miscarriage (Pfeiffer *et al.*, 2001). Thus, these combined results indicate that *HLA-G**0105N, which is a null allele for the HLA-G1 isoforms, is a risk factor for recurrent miscarriage. In addition, our study suggests that the *HLA-G**0104 allele also confers risk, further implicating a critical role for the G1 isoforms in maintenance of pregnancy.

We did not confirm the recent report of an association with the *HLA-G**01013 allele (Pfeiffer *et al.*, 2001). The *01013 allele differs from other *0101 alleles by silent variation in exons 2 and 3 and does not encode a different protein. Thus, it is likely that the *01013 allele is a marker for additional variation on the same haplotype with the *01013 allele in their population. This could be due to variation in the promoter region, as suggested in one published studey (Pfeiffer *et al.*, 2001). We sequenced the HLA-G promoter and upstream control region for polymorphisms in the subjects in this study and found at least three different promoter haplotypes associated with the *01013 allele (Aldrich, 2001). It is possible that the predominant promoter region haplotype associated with *01013 in the German study differs from the predominant haplotypes in our sample, thus accounting for the differences in our results. Alternatively, differences could be due to sample heterogeneity or differences in study design. For example, our study excluded couples that did not become pregnant within 12 months following enrolment (Ober *et al.*, 1999).

One documented function of HLA-G1 is its interaction with natural killer (NK) cell inhibitory receptors (Pazmany *et al.*, 1996; Hiby *et al.*, 1997; Rajagopalan and Long, 1999; Sasaki *et al.*, 1999). However, the HLA-G1 isoforms have also been shown to inhibit T cells in several ways including induction of apoptosis (Fournel *et al.*, 2000), reduction of cytotoxicity in a concentration-dependent manner (Kapasi *et al.*, 2000) and suppression of the allo-proliferative response (Bainbridge *et al.*, 2000b). Thus, it is possible that the amino acid change at position 110 decreases the efficiency with which HLA-G binds to and/or inhibits NK or T cells. The decreased expression of the HLA-G1 isoform in carriers of the *0105N allele could have the same general result of reduced inhibition of NK and/or T cells in the placenta. While there are, at present, no studies of the specific residues of HLA-G that interact with

NK inhibitory receptors, the analogous residue (at amino acid 110) in HLA-C does not contact the NK receptor that is specific for HLA-C (Boyington *et al.*, 2000). Likewise, there have been no studies to date that characterize the interaction of specific HLA-G motifs with receptors on the T cell. With so little information about the specific amino acid residues that are critical for inhibition of either NK cells or T cells, it is difficult to assess the impact of the *0104 allele on these interactions. Alternatively, this polymorphism may be in linkage disequilibrium with other variants in this or nearby genes. However, it seems unlikely that other variation in *HLA-G* accounts for our findings given the overall lack of polymorphism in the coding region of *HLA-G* (Hviid *et al.*, 1997; Ober *et al.*, 1998; van der Ven *et al.*, 1998; Alvarez *et al.*, 1999; Hiby *et al.*, 1999; Loke *et al.*, 1999) and the fact that variants in the promoter and upstream control regions showed no association with pregnancy outcome in this sample (Aldrich, 2001). We cannot exclude the possibility that these effects are due to linkage disequilibrium with variants in a nearby gene, but this would require that the *0104 and *0105N alleles are each in linkage disequilibrium with alleles that are associated with miscarriage.

In this study, the presence of *HLA-G**0104 and *HLA-G**0105N alleles was unrelated to efficacy of treatment with paternal mononuclear cell immunization. Therefore, the *HLA-G* genotype of couples with a history of recurrent miscarriage would not identify a subgroup of patients that would benefit from this treatment. Nonetheless, the presence of the *0104 and *0105N alleles may identify a subset of patients with a specific genetic aetiology for their pregnancy losses. On the other hand, the presence of the *0104 and *0105N alleles in the fetus does not preclude successful outcomes as both alleles are found commonly in healthy adult populations (Ober *et al.*, 1996, 1998; Suarez *et al.*, 1997; Ober and Aldrich, 1997; Castro *et al.*, 2000). Although the role of different HLA-G isoforms in pregnancy is still poorly understood, these data suggest that the HLA-G1 isoform may play an important role in establishing and maintaining the fetal–placental unit in early pregnancy. Whether this is a result of interactions with NK cells, alloreactive T cells or other, as yet undefined, mechanisms is unknown. Further studies of *HLA-G* alleles in couples with recurrent miscarriage are needed to further elucidate the mechanism by which the HLA-G1 proteins facilitate successful pregnancy outcome. Nonetheless, our study and that of Pfeiffer *et al.* (Pfeiffer *et al.*, 2001) establish a role for HLA-G in the maintenance of successful pregnancy.

Acknowledgement

This work was supported by NIH grants HD27626 and HD21244.

References

Aldrich, C.L. (2001) Clinical and Molecular Evolutionary Studies of the Nonclassical Human Leukocyte Antigen, HLA-G. *Committee on Genetics.* The University of Chicago, Chicago.

Aldrich, C., Verp, M.S., Walker, M.A. *et al.* (2000) A null mutation in HLA-G is not associated with preeclampsia or intrauterine growth retardation. *J. Reprod. Immunol.*, **47**, 41–48.

Alvarez, M., Santos, P., Martinho, A. *et al.* (1999) HLA-G genetic polymorphism in 57 Portuguese white families studied by PCR-RFLP and PCR-SSOP. *Transplant. Proc.*, **31**, 1829–1831.

Bainbridge, D.R., Ellis, S.A. and Sargent, I.L. (2000a) The short forms of HLA-G are unlikely to play a role in pregnancy because they are not expressed at the cell surface. *J. Reprod. Immunol.*, **47**, 1–16.

Bainbridge, D.R., Ellis, S.A. and Sargent, I.L. (2000b) HLA-G suppresses proliferation of CD4(+) T-lymphocytes. *J. Reprod. Immunol.*, **48**, 17–26.

Boyington, J.C., Motyka, S.A., Schuck, P. *et al.* (2000) Crystal structure of an NK cell immunoglobulin-like receptor in complex with its class I MHC ligand. *Nature*, **405**, 537–543.

Cao, W., Brenner, C.A., Alikani, M. *et al.* (1999) Search for a human homologue of the mouse *Ped* gene. *Mol. Hum. Reprod.*, **5**, 541–547.

Castro, M.J., Morales, P., Rojo-Amigo, R. *et al.* (2000) Homozygous HLA-G*0105N healthy individuals indicate that membrane-anchored HLA-G1 molecule is not necessary for survival. *Tissue Antigens*, **56**, 232–329.

Ellis, S.A., Palmer, M.S. and McMichael, A.J. (1990) Human trophoblast and the choriocarcinoma cell line BeWo express a truncated HLA Class I molecule. *J. Immunol.*, **144**, 731–735.

Ellis, S.A., Sargent, I.L., Redman, C.W. *et al.* (1986) Evidence for a novel HLA antigen found on human extravillous trophoblast and a choriocarcinoma cell line. *Immunology*, **59**, 595–601.

Fournel, S., Aguerre-Girr, M., Huc, X. *et al.* (2000) Cutting Edge: soluble HLA-G1 triggers CD95/CD95 ligand-mediated apoptosis in activated CD8+ cells by interacting with CD8. *J. Immunol.*, **164**, 6100–6104.

Hara, N., Fujii, T., Yamashita, T. *et al.* (1996) Altered expression of human leukocyte antigen G (HLA-G) on extravillous trophoblasts in preeclampsia: immunohistological demonstration with anti-HLA-G specific antibody '87G' and anti-cytokeratin antibody 'CAM5.2'. *Am. J. Reprod. Immunol.*, **36**, 349–358.

Hashimoto, K., Azuma, C., Koyama, M. *et al.* (1997) Biparental alleles of HLA-G are co-dominantly expressed in the placenta. *Jap. J. Hum. Genet.*, **42**, 181–186.

Hiby, S.E., King, A., Sharkey, A.M. *et al.* (1997) Human uterine NK cells have a similar repertoire of killer inhibitory and activatory receptors to those found in blood, as demonstrated by RT–PCR and sequencing. *Mol. Immunol.*, **34**, 419–430.

Hiby, S.E., King, A., Sharkey, A. *et al.* (1999) Molecular studies of trophoblast HLA-G: polymorphism, isoforms, imprinting and expression in preimplantation embryo. *Tissue Antigens*, **53**, 1–13.

Houlihan, J.M., Biro, P.A., Harper, H.M. *et al.* (1995) The human amnion is a site of MHC class Ib expression: evidence for the expression of HLA-E and HLA-G. *J. Immunol.*, **154**, 5665–5674.

Hunt, J.S. and Orr, H.T. (1992) HLA and maternal–fetal recognition. *FASEB J.*, **6**, 2344–2348.

Hunt, J.S., Jadhav, L., Chu, W. *et al.* (2000) Soluble HLA-G circulates in maternal blood during pregnancy. *Am. J. Obstet. Gynecol.*, **183**, 682–688.

Hviid, T.V., Meldgaard, M., Sorensen, S. *et al.* (1997) Polymorphism of exon 3 of the *HLA-G* gene. *J. Reprod. Immunol.*, **35**, 31–42.

Ishitani, A. and Geraghty, D.E. (1992) Alternative splicing of HLA-G transcripts yields proteins with primary structures resembling both class I and class II antigens. *Proc. Natl Acad. Sci. USA*, **89**, 3947–3951.

Kapasi, K., Albert, S.E., Yie, S. *et al.* (2000) HLA-G has a concentration-dependent effect on the generation of an allo-CTL response. *Immunology*, **101**, 191–200.

Karhukorpi, J., Laitinen, T. and Tiilikainen, A.S. (1997) HLA-G polymorphism in Finnish couples with recurrent spontaneous miscarriage. *Br. J. Obstet. Gynaecol.*, **104**, 1212–1214.

King, A., Boocock, C., Sharkey, A.M. *et al.* (1996) Evidence for the expression of HLAA-C class I mRNA and protein by human first trimester trophoblast. *J. Immunol.*, **156**, 2068–2076.

Kovats, S., Main, E.K., Librach, C. *et al.* (1990) A class I antigen, HLA-G, expressed in human trophoblasts. *Science*, **248**, 220–223.

Le Bouteiller, P. and Blaschitz, A. (1999) The functionality of HLA-G is emerging. *Immunol. Rev*, **167**, 233–244.

Lim, K.H., Zhou, Y., Janatpour, M. *et al.* (1997) Human cytotrophoblast differentiation/invasion is abnormal in pre-eclampsia. *Am. J. Pathol.*, **151**, 1809–1818.

Loke, Y.W., Hiby, S. and King, A. (1999) Human leucocyte antigen-G and reproduction. *J. Reprod. Immunol.*, **43**, 235–242.

Mallet, V., Proll, J., Solier, C. *et al.* (2000) The full length HLA-G1 and no other alternative form of FLA-G is expressed at the cell surface of transfected cells. *Hum. Immunol.*, **61**, 212–224.

Mehta, C.R. and Patel, N.R. (1983) A network algorithm for performing Fisher's exact test in rxc tables. *J. Am. Stat. Assoc.*, **76**, 931–934.

Ober, C. and Aldrich, C.L. (1997) HLA-G polymorphisms: neutral evolution or novel function? *J. Reprod. Immunol.*, **36**, 1–21.

Ober, C., Rosinsky, B., Grimsley, C. *et al.* (1996) Population genetic studies of HLA-G: allele frequencies and linkage disequilibrium with HLA-A1. *J. Reprod. Immunol.*, **32**, 111–123.

Ober, C., Aldrich, C., Rosinsky, B. *et al.* (1998) HLA-G1 protein expression is not essential for fetal survival. *Placenta*, **19**, 127–132.

Ober, C., Karrison, T., Odem, R.R. *et al.* (1999) Mononuclear-cell immunisation in prevention of recurrent miscarriages: a randomised trial. *Lancet*, **354**, 365–369.

Pazmany, L., Mandelboim, O., Vales-Gomez, M. *et al.* (1996) Protection from natural killer cell-mediated lysis by HLA-G expression on target cells. *Science*, **274**, 792–795.

Penzes, M., Rajczy, K., Gyodi, E. *et al.* (1999) HLA-G gene polymorphism in the normal population and in recurrent spontaneous abortion in Hungary. *Transplant. Proc.*, **31**, 1832–1833.

Pfeiffer, K.A., Fimmers, R., Engels, G. *et al.* (2001) The HLA-G genotype is potentially associated with idiopathic recurrent spontaneous abortion. *Mol. Hum. Reprod.*, **7**, 373–378.

Rajagopalan, S. and Long, E.O. (1999) A human histocompatibility leukocyte antigen (HLA)-G-specific receptor expressed on all natural killer cells. *J. Exp. Med.*, **189**, 1093–1100.

Rebmann, V., Van Der Ven, K., Päβler, M. *et al.* (2000) Association of soluble HLA-G plasma levels with *HLA-G* alleles. *Tissue Antigens*, **57**, 15–21.

Redman, C.W., McMichael, A.J., Stirrat, G.M. *et al.* (1984) Class 1 major histocompatibility complex antigens on human extra-villous trophoblast. *Immunology*, **52**, 457–468.

Sasaki, H., Xu, X.C., Smith, D.M. *et al.* (1999) HLA-G expression protects porcine endothelial cells against natural killer cell-mediated xenogeneic cytotoxicity. *Transplantation*, **67**, 31–37.

Schmidt, C.M. and Orr, H.T. (1993) Maternal/fetal interactions: the role of the MHC class I molecule HLA-G. *Crit. Rev. Immunol.*, **13**, 207–224.

Suarez, M.B., Morales, P., Castro, M.J. *et al.* (1997) A new *HLA-G* allele (*HLA-G**0105N) and its distribution in the Spanish population. *Immunogenetics*, **45**, 464–465.

van der Ven, K., Skrablin, S., Engels, G. *et al.* (1998) HLA-G polymorphisms and allele frequencies in Caucasians. *Hum. Immunol.*, **59**, 302–312.

Wei, X.H. and Orr, H.T. (1990) Differential expression of HLA-E, HLA-F, and HLA-G transcripts in human tissue. *Hum. Immunol.*, **29**, 131–142.

Wilson, R.D., Kendrick, V., Wittmann, B.K. *et al.* (1984) Risk of spontaneous abortion in ultrasonically normal pregnancies. *Lancet*, **ii**, 920–921.

Wooding, F.B.P. and Flint, A.P.F. (1994) Placentation. In Lamming, G.E. and Parkes, A. (eds) *Marshall's Physiology of Reproduction*, Vol. 3. Chapman and Hall, London, pp. 233–460.

Yamashita, T., Fujii, T., Tokunaga, K. *et al.* (1999) Analysis of human leukocyte antigen-G polymorphism including intron 4 in Japanese couples with habitual abortion. *Am. J. Reprod. Immunol.*, **41**, 159–163.

Received on April 30, 2001; accepted on September 26, 2001