Comparison of the expression of human leukocyte antigen (HLA)-G and HLA-E in women with normal pregnancy and those with recurrent miscarriage

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Abstract

Recurrent miscarriage affects 1% of all couples attempting pregnancy. Immunological factors are postulated to play a role in the aetiology of recurrent miscarriage because the fetus and placenta are immunologically different from the mother. In particular, altered expression of the, non-classical, class I histocompatibility leukocyte antigen (HLA) molecules has been postulated to play a role in the aetiology of recurrent miscarriage as the fetus and placenta are semi-allogenic to the mother. This study was conducted to examine whether altered expression of the non-classical class I HLA molecules, HLA-G and HLA-E, by cells at the maternofetal interface could play a role in the aetiology of recurrent miscarriage. First-trimester placental and decidual biopsies were obtained from 45 women with recurrent miscarriage and 17 gestation-matched normal controls. These biopsies were screened by immunohistochemistry for HLA-G and HLA-E and isotype-matched control antibodies. Staining was analysed by light microscopy and digital image analysis. In both recurrent miscarriage and normal pregnancy, HLA-G was localised to the extravillous trophoblast. There was no difference in the pattern of HLA-G expression between women with recurrent miscarriage and those with normal pregnancies. HLA-E was localised to the syncytiotrophoblast, villous mesenchymal cells, extravillous trophoblast and several decidual cell types, but staining for HLA-E appeared to be confined primarily to the cytoplasm. There was no difference in the pattern of HLA-E expression between women with recurrent miscarriage and those with normal pregnancies.

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Introduction

Recurrent miscarriage (RM) is commonly defined as three or more consecutive first-trimester or two or more consecutive second-trimester pregnancy losses before 20 weeks' gestation. Using this definition, 1–2% of couples experience RM (Coulam 1991). The risk of a spontaneous miscarriage in clinically identified pregnancy is estimated to be approximately 12–14% (Edmonds *et al.* 1982). Consequently, the incidence of three miscarriages by chance alone is in the order of 0.34% (Stirrat 1990a). This discrepancy implies that, in addition to random causes, some couples are at an increased risk of RM which suggests a specific underlying pathology. Anatomical, infectious, endocrine and genetic factors are all known to be associated with RM. However, the aetiology of RM remains unknown in approximately 50% of cases (Lee & Silver 2000).

Potential immunological causes of otherwise unexplained RM have generated considerable interest and

controversy. A prominent hypothesis in the area of immune-related RM is that some cases of RM are due to a maternal immune response to the fetus and placenta. This hypothesis holds that the placenta is in effect a foreign tissue (transplant), and that, normally, mechanisms are in place to prevent a maternal immune response to this foreign tissue. It is hypothesised that in some cases of RM these regulatory mechanisms fail, allowing the maternal immune system to respond to fetal antigens. One mechanism that might limit maternal immune responses is immunosuppression of the populations of leukocytes present at the maternofetal interface. During the first trimester of pregnancy the human decidua is rich in leukocytes which make up 10–15% of all decidual cells (Loke & King 1995). This leukocyte population is composed of up to 70% uterine natural killer (NK) cells and 10% T cells (Loke & King 1995).

Immunosuppression of these leukocytes is believed to be mediated, in part, by the non-classical class I histocompatibility leukocyte antigen (HLA) molecule, HLA-G. HLA-G

has been shown to bind to the immunoglobulin-like transcript (ILT)-2 and killer inhibitory receptor (KIR)2DL4 (p49) inhibitory receptors on NK cells and may confer protection to extravillous trophoblasts (EVTs) via these receptors (Allan *et al.* 1999, Biassoni *et al.* 1999). Recently, a second non-classical class I HLA molecule, HLA-E, has been shown to be expressed by trophoblasts and HLA-E is also believed to help the fetus to avoid maternal immune surveillance, possibly by interacting with the CD94/NKG2A NK-cell inhibitory receptor (Braud *et al.* 1998, Lee *et al.* 1998).

This investigation was undertaken in order to determine whether there was an altered expression of HLA-G or HLA-E at the maternofetal interface in women with RM compared with women with normal pregnancies.

Materials and Methods

Tissues

This study was approved by the Auckland Regional Health and Disability Ethics Committee and all tissue samples were obtained with informed consent.

Collection of the specimens has been described previously (Askelund *et al.* 2004); briefly, decidual biopsies from the implantation site and/or villous placental samples were collected from 45 women attending the RM clinic at the National Women's Hospital by ultrasound-guided curettage as soon as possible after confirming embryonic/fetal demise. This usually occurred within 6 h of the confirmation of fetal demise (Fig. 1). In all cases this was prior to the onset of vaginal bleeding.

Women attending the RM clinic were extensively investigated and monitored closely. Routine investigations included full blood count, rubella, hepatitis B and syphilis serology; and blood group and antibody screen. In addition, women were screened for antinuclear antibodies and extractable nuclear antigens, anticardiolipin antibodies and lupus anticoagulant. Women were also investigated for activated protein C resistance and if this analysis was abnormal they were screened for Factor V Leiden mutations. All women had swabs for bacterial vaginosis. Quantitative β -human chorionic gonadotrophin (β HCG) assays were performed three times weekly until 5 weeks

of gestation and then weekly until 9 weeks of gestation. Ultrasound scans were performed weekly from 6 gestational weeks. Consequently, early pregnancy failure was generally predicted by either an abnormal trend in serial β HCG measurements or ultrasound findings. As soon as embryonic or fetal demise was confirmed, evacuation of the uterus was arranged. The method of ultrasound-guided biopsy has been described previously (Khong *et al.* 1987). All products of conception were analysed for fetal karyotype, 15 were of normal karyotype, 5 failed to grow and therefore could not be karyotyped and the remaining pregnancies had abnormal karyotypes. In all cases, gestational age assessment was based on crown rump length measurement by ultrasound. Gestation-matched decidual biopsies and/or villous placental tissues were also collected from 17 women undergoing an elective surgical termination of pregnancy (TOP) (Fig. 1). The gestational age and fetal viability of all pregnancies in this group were confirmed by ultrasound assessment. In no cases in this group was there vaginal bleeding or evidence of embryonic or fetal demise prior to the termination of pregnancy.

All samples were embedded in cryoembedding compound (TBS, Biotek Auckland, New Zealand) and snap frozen in liquid nitrogen-cooled 2-methyl butane then stored at -80°C until required. Serial thin sections ($5\text{ }\mu\text{m}$) were cut using a cryostat (Leica CM1900, Germany), fixed for 10 min in cold acetone, air-dried, then wrapped in aluminum foil and parafilm and stored at -20°C until use.

Immunohistochemistry

Slides were thawed and non-specific binding sites were blocked by the addition of $100\text{ }\mu\text{l}$ 10% normal goat serum (blocking solution) (Life Technologies, Auckland, New Zealand), diluted in PBS, pH 7.4, containing 0.05% Tween 20 (PBS-Tween) (Serva, Heidelberg, Germany) for 10 min. The slides were then washed three times in PBS-Tween for 2 min. Following this, the slides were incubated with $50\text{ }\mu\text{l}$ of the primary antibody in 10% normal goat serum in PBS-Tween for 1 h at room temperature. The HLA-G antibody, MEM-G/9 (Abcam, Cambridge, UK) was used at a dilution of 1:200 and the HLA-E antibody, MEM-E/02 (Abcam), was used at a dilution of 1:300. Both MEM-G/9 and MEM-E/02 are IgG1 isotype antibodies. The slides were then washed three times in PBS-Tween for 2 min per wash and incubated with $50\text{ }\mu\text{l}$ 5% H_2O_2 in methanol for 5 min to quench endogenous peroxidase activity. The slides were then washed three times in PBS-Tween (2 min/wash) and incubated with $50\text{ }\mu\text{l}$ biotinylated (goat) secondary antibody (broad-spectrum staining kit; Zymed, San Francisco, CA, USA) for 10 min at room temperature. Following this the slides were washed again in PBS-Tween and incubated with $50\text{ }\mu\text{l}$ streptavidin-conjugated horse radish peroxidase (Zymed) for 10 min at room temperature. The slides were washed a further three times with PBS-Tween (2 min/wash) and incubated with

Figure 1 Samples for this study were obtained from a total of 62 women and are presented by gestational age. Women with RM (black bars); women undergoing termination of pregnancy (grey bars).

50 µl of the chromagen 3-amino-9-ethylcabazole (AEC) (Dako, MedBio, Christchurch, New Zealand) for 20 min at room temperature. Finally, the slides were washed for 2 min in deionised water and counterstained with Gills No. 2 haematoxylin (Amber Scientific, Australia) for 30 s. The slides were then washed in tap water and coverslips (BioLab Scientific, Auckland, New Zealand) mounted using Aquamount (BHD, Palmerston North, New Zealand). Additional slides were stained with IgG1 isotype antibodies reactive with cytokeratin, CD45, CD3, CD56 (Dako), CD57 (IgM, Novo Castra, Newcastle upon Tyne, UK) and CD14 (IgG2b isotype, this laboratory) using the same method. The semi-serial sections were used as isotype-matched controls and also to assist in the identification of cell types within the tissues.

Analysis of HLA-G and HLA-E immunostaining

Sections were analysed for HLA-G and HLA-E staining using a light microscope (Nikon ECLIPSE E400, Japan) and photomicrographs were taken using a Nikon Coolpix 990 digital camera (Nikon). EVT's were identified based on cytokeratin staining in semi-serial sections. Cells immunostained for HLA-E were identified based on morphology and staining patterns seen in semi-serial sections stained with HLA-G, cytokeratin, CD45 and CD14.

The HLA-G and HLA-E staining was analysed descriptively. HLA-G staining in EVT's was then analysed semi-quantitatively by grading the intensity of HLA-G expression on a scale of 0 to 3 (where: 0 = no staining; 1 = light staining intensity; 2 = medium staining intensity; 3 = strong staining intensity). Statistical significance of differences between groups was assessed using the Wilcoxon rank sum test. All differences were considered to be statistically significant when the *P* value was ≤ 0.05 .

HLA-E expression was further analysed by determining the percentage of those samples in which specific cell types stained for HLA-E. Whether differences were statistically significant was assessed using Fisher's exact test for percentages. All differences were considered to be statistically significant when the *P* value was ≤ 0.05 .

Results

HLA-G expression

In the trophoblast columns HLA-G immunostaining was localised to EVT's from women with normal pregnancies and those with RM while there was no staining of any cell type in the villi. Furthermore, the staining intensity of HLA-G increased as EVT's migrated further away from the villi (Fig. 2). There were two samples from women with normal pregnancies, as well as two samples from women with RM, that contained trophoblast columns that did not stain for HLA-G (Fig. 2). There was strong immunostaining for HLA-G in EVT's that had invaded the decidua, including endovascular trophoblast (Fig. 2). There were no obvious

differences in the expression pattern of HLA-G between women with RM and those with normal pregnancies.

Semi-quantitative analysis revealed that the mean HLA-G staining intensity in the proximal trophoblast columns from women with RM (2.1) was not significantly different ($P = 0.051$) from that in women with normal pregnancies (1.55). Likewise, there was no significant difference in the staining intensity of HLA-G in decidual EVT's between women with RM and those with normal pregnancies ($P = 0.072$).

We also compared the staining intensity of HLA-G, in a subgroup of women with RM whose products of conception were karyotypically normal (2.31), with the HLA-G staining intensity in the women with normal pregnancies (1.55) but found no significant difference ($P = 0.069$).

HLA-E expression

In general there was no staining for HLA-E in the villi. However, very rarely the cytoplasm of syncytiotrophoblast in sporadic villi stained strongly positive for HLA-E (Fig. 3F). EVT's in some, but not all, proximal columns and placental septae stained strongly for HLA-E (Fig. 3B).

HLA-E immunostaining was localised to some interstitial trophoblast in the decidual stroma. Strong HLA-E immunostaining was found on large decidualised stromal cells and uterine epithelium (Fig. 3C), uterine endothelial cells also stained. In all of these three cell types HLA-E appeared to be limited to the cytoplasm but a few large decidualised stromal cells exhibited a rim pattern of staining. Other decidual stromal cells also stained positive for HLA-E (Fig. 3C) and based on their morphology and the staining patterns observed in semi-serial sections stained with CD56, CD3 or CD14, these cells were thought to be leucocytes.

Semi-quantitative analysis revealed that there was no significant difference in the expression of HLA-E by EVT's in the proximal columns (68% vs 58%; $P = 0.072$) or in the decidua (33% vs 72%; $P = 0.10$) between women with RM and those with normal pregnancies respectively.

Comparison of the expression of HLA-E and HLA-G revealed that 35% of the samples contained EVT's in the proximal trophoblast columns that expressed HLA-G but not HLA-E (Fig. 2A and B). Furthermore, one specimen contained EVT's in a proximal column that were immunostained for HLA-E in the absence of HLA-G immunostaining (Fig. 3A and B).

Discussion

HLA-G is a, non-classical, class I HLA molecule, exhibiting low polymorphism that is postulated to mediate, in part, maternal tolerance to the semi-allogenic fetus by acting as an invariant 'fetal' signal (Loke & King 1997, Stirrat 1990b). HLA-G may perform this function by interacting with the NK-cell inhibitory receptors ILT-2 and the KIR2DL4 (p49) receptor found on uterine NK cells (Biassoni *et al.* 1999),

Figure 2 Photomicrographs depicting the immunohistochemical localisation of HLA-G and HLA-E in the placenta and decidua. (A) Gradient in the staining of HLA-G expression in a proximal trophoblast column. (B) Lack of expression of HLA-E in a section semi-serial to panel A. (C) Localisation of HLA-G in endovascular trophoblast (arrow) and decidual EVTs. (D) Section semi-serial to panel C stained with negative control anti-CD57 (arrow indicates endovascular trophoblast). (E) EVTs in a proximal column showing no HLA-G staining (arrow).

as well as with the T-cell receptor, to inhibit NK-mediated cytotoxicity and allogeneic T-cell responses respectively (Riteau *et al.* 1999). Further evidence for the role of HLA-G in mediating maternofetal tolerance is that it is only expressed by EVTs (Loke & King 1995). EVTs are the only fetal cells that invade the decidua thereby coming into direct contact with cells of the maternal decidua immune system (Kovats *et al.* 1990, Wei & Orr 1990).

The results of this study are in accordance with previous studies showing that the membrane-bound HLA-G isoform is only expressed by EVTs. In addition, there is a gradient

of staining, such that as EVTs migrate further away from the villi and into the decidua, they increase their expression of HLA-G. Most EVTs express HLA-G and this expression appeared consistent with localisation to the cell membrane. EVTs that had invaded the decidua strongly expressed HLA-G in both women with normal pregnancies and those with RM. This finding excludes the possibility that RM could be explained solely by the lack of HLA-G expression by EVTs.

Semi-quantitative analysis revealed that there was no statistically significant difference in the mean intensity of

Figure 3 Photomicrographs depicting the immunohistochemical localisation of HLA-E in the placenta and decidua. Semi-serial sections through a proximal trophoblast column were stained for (A) HLA-G and (B) HLA-E; while some trophoblasts in this column expressed both HLA-G and HLA-E (double-ended arrow) other trophoblasts expressed HLA-E but not HLA-G (arrows). (C) HLA-E was also localised to large decidua stromal cells (large arrows) and decidua leucocytes (small arrow). (D) Section semi-serial to panel C stained with an isotype-matched control antibody reactive with CD56. (E) HLA-E expression in glandular epithelium; (F) HLA-E was localised to sporadic areas of syncytiotrophoblast (arrow).

HLA-G staining by EVT cells between women with normal pregnancies or with RM. This result is consistent with one study which also found no difference in the expression of HLA-G between women with RM and with normal pregnancies (Patel *et al.* 2003), but is contrary to the findings of Emmer *et al.* (2002) who observed a lower intensity of HLA-G staining of decidual EVT cells from women with RM compared with those from women with normal pregnancies. However, Emmer *et al.* (2002) examined samples from only two normal pregnancies and nine women with RM; such a sample size is likely to be inadequate to allow valid conclusions to be drawn. Thus, results from this study suggest that RM is not caused by a lack of HLA-G expression by EVT cells. This finding is supported by a previous report of a woman who was homozygous for the HLA-G null allele (Ober *et al.* 1998), who had survived life *in utero*, as a fetus whose placenta could not express HLA-G, for 9 months. While this study suggests that RM is not associated with a lack of expression of HLA-G protein by EVT cells, it remains possible that the HLA-G protein expressed by EVT cells in women with RM may be functionally defective.

In this study (using the MEM-G/09 antibody) we did not detect HLA-G on placental endothelial cells or other villous stromal cell types. Other workers have reported that HLA-G is localised to fetal endothelial cells both in the first trimester and at term (Blaschitz *et al.* 1997, 2001, Dye *et al.* 2001). However, detection of HLA-G on placental endothelial cells appears to be dependent upon the specific epitope within HLA-G recognised by individual monoclonal antibodies.

EVT cells have been shown to express the, non-classical, class I HLA-E protein (Wei & Orr 1990, King *et al.* 2000, Ishitani *et al.* 2003). HLA-E may also be perceived as a 'self' signal by maternal NK cells expressing the inhibitory receptor CD94/NKG2A, thus collaborating with HLA-G to prevent cytotoxicity (King *et al.* 2000). Results from this study demonstrate that, in accordance with previous studies, HLA-E is expressed by EVT cells. However, in contrast to the findings of Ishitani *et al.* (2003), villous cytotrophoblasts were not found to express HLA-E and while we did find expression of HLA-E by some syncytiotrophoblasts, this rare staining was not typical. We also found that in the decidua, HLA-E is expressed by large decidualised stromal cells, glandular epithelium, uterine endothelial cells and by decidual leukocytes.

Cell surface expression of any class I HLA molecule requires the formation of a trimolecular complex consisting of the HLA class I heavy chain, β microglobulin, and the peptide antigen that will be presented to T cells. In the case of HLA-E, these peptides are derived from the N-terminal signal sequences of some HLA class I molecules, especially that of HLA-G but also HLA-C (Braud *et al.* 1997, Lee *et al.* 1998, Navarro *et al.* 1999). Binding of leader peptides from MHC class I molecules stabilises the HLA-E protein and allows it to migrate to the cell surface where it can interact with the NK-cell inhibitory receptor CD94/NKG2A. In EVT cells

cell surface expression of HLA-E is thought to be primarily dependent on HLA-G expression, although HLA-C may also be involved. Syncytiotrophoblast and villous mesenchymal cells do not express the transmembrane form of HLA-G but do express, in a limited fashion, HLA-E. This suggests that HLA-E expression might be dependent on the expression of the soluble form of HLA-G1 which is reportedly expressed by syncytiotrophoblasts (Ishitani *et al.* 2003). Others have shown that the additional splice variants of HLA-G can not support the cell surface expression of HLA-E (Ulbrecht *et al.* 2004).

Ishitani *et al.* (2003) reported that all cells expressing HLA-G expressed HLA-E. While we found that all cell types expressing HLA-G also expressed HLA-E, we did find that a number of individual cells, in particular decidual EVT cells, expressed HLA-G but not HLA-E. The fact that some cells did not coexpress HLA-E with HLA-G suggests that HLA-G expression is not sufficient to ensure expression of HLA-E in EVT cells. Others have recently suggested that a similar situation occurs in tumour cell lines, where the presence of class I HLA-A or HLA-C proteins that should allow HLA-E expression does not necessarily lead to expression of HLA-E on the cell surface (Palmisano *et al.* 2005).

In an immunohistochemical study such as ours it is not possible to determine whether immunoreactive proteins are expressed at the cell surface or not. However, HLA-E reactivity in most cell types, including EVT cells, appeared to be limited to the cytoplasm and did not appear to be associated with the cell membrane. This suggests that the HLA-E we detected in many cells was not expressed on the cell surface. This leads us to question the functional significance of the apparent expression of HLA-E at the maternofetal interface.

In agreement with others (Wei & Orr 1990, Blaschitz *et al.* 2001, Ishitani *et al.* 2003) we found a number of cell types that appeared to express HLA-E, but the staining for HLA-E was often inconsistent. It is important to stress this point because, while HLA-E is expressed by a variety of cell types in the placenta and decidua, not all of the cells of a given type express HLA-E. For example, some small regions of syncytiotrophoblast express HLA-E while the majority of the syncytiotrophoblast does not. The reason why the staining for HLA-E is not uniform remains unclear and leads us again to question the functional significance of HLA-E expression. In contrast to our results, others, using different antibodies, have reported no expression of HLA-E in any trophoblast cell population (Blaschitz *et al.* 2001); however, this conflict could be explained by the epitope specificity of the different antibodies.

One limitation of this and similar studies is that the small sample size may mean that the study was underpowered to detect important differences in the expression of HLA molecules between RM and normal pregnancy. However, the fact that the two larger studies conducted to date, ours and that of Patel *et al.* (2003), both found no

difference in the expression of HLA-G between RM and normal pregnancy suggests that this is not the case.

In conclusion, we have shown that there is no significant difference in the expression patterns of HLA-G or HLA-E at the maternofetal interface in women with RM compared with women with normal pregnancies. We have also shown that expression of HLA-G is not sufficient to ensure co-expression of HLA-E and we question the functional significance of HLA-E at the maternofetal interface.

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