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An alternatively spliced form of *HLA-G* mRNA in human trophoblasts and evidence for the presence of *HLA-G* transcript in adult lymphocytes

(nonclassical class I major histocompatibility complex antigen/first trimester trophoblasts/adult peripheral blood)

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ABSTRACT **The *HLA-G* monomorphic, nonclassical class I gene encodes the major histocompatibility complex (MHC) molecule, which is the only MHC antigen expressed on cytotrophoblast cells of placenta. In this work, we have investigated expression of the *HLA-G* gene in fetal tissues and adult peripheral blood cells by using a sensitive hot-start reverse transcriptase PCR technique. PCR amplification with *HLA-G* primers specific for exon 3 has enabled us to demonstrate an alternatively spliced form of *HLA-G* mRNA present in fetal first trimester trophoblasts and lacking exon 4 (*HLA-G.3–5*). This low abundance transcript (≈1:200) in comparison to full-length mRNA may encode the protein that excludes the α3 domain and by conformational changes may present a different ability to bind to peptides. Moreover, expression of the *HLA-G* transcript was found in adult peripheral lymphocytes and equally in B- and T-cell populations. These results are discussed in the context of the fetal–maternal relationship presented by *HLA-G* gene products.**

The human major histocompatibility complex (MHC) is located in the distal region of the short arm of chromosome 6 (6p21.3) and spans a genomic region of ≈4000 kb. This region includes three subregions of MHC genes: class I, class II, and class III. The class I subregion is larger than the two others, spanning 2000 kb, and is composed of >20 genes and pseudogenes (1). This class I region comprises two groups of genes: (*i*) Classical HLA class I genes (*HLA-A*, *HLA-B*, *HLA-C*), highly polymorphic and ubiquitously expressed, encode cell surface 45-kDa glycoproteins associated with an invariant 12-kDa β_2-microglobulin chain. These antigens constitute the restriction elements for virus-specific and allospecific cytolytic T lymphocytes. (*ii*) Nonclassical HLA class I genes (*HLA-E*, *HLA-F*, *HLA-G*) whose functions are not yet established, but it is possible that each of these genes has a specific function (2). Among nonclassical HLA class I genes, *HLA-E* and *HLA-F* are expressed in many fetal and adult tissues (3–5). *HLA-G* nonpolymorphic antigen is expressed only in fetal placental tissues and liver. In the first trimester, high expression was detected in placental cells such as the extravillous trophoblast populations and different cytotrophoblast cells (2, 6–9) but not in extravillous membrane (2). In the third trimester this expression is reduced in extravillous cytotrophoblast and increased in extravillous membrane (2, 7, 8). In fetal liver the *HLA-G* gene is expressed in the first trimester but not in the second trimester (10). Moreover, *HLA-G* mRNA was also detected in choriocarcinoma cell lines (6, 11). *HLA-G* gene sequence and structure are tightly homologous to classical HLA class I genes (12, 13) and other nonclassical HLA class I genes (3, 4); however, due to the stop codon in exon 6, the *HLA-G* gene encodes the protein with a shorter cytoplasmic tail than classical HLA class I genes (13). Nevertheless, HLA-G antigen appears to be nonpolymorphic; the *HLA-G* gene presents several heterogeneities of sequences as single base substitutions in the coding regions without modifications of protein sequence, single base substitutions in introns, and a 14-base insertion in the 3′ untranslated region (14). Moreover, three different forms of alternatively spliced mRNA were observed—i.e., full-length mRNA transcript and transcripts lacking exon 3 or exons 3 and 4 (15). In all but one study (16), no expression of *HLA-G* was found in syncytiotrophoblasts. In adults the expression of *HLA-G* was observed in eye tissue (15). Specific expression of the *HLA-G* gene restricted to fetal trophoblasts, the only fetal tissue in contact with maternal cells, which lack the classical MHC class I antigens (HLA-A, -B, -C), may suggest the role of this gene in maternal tolerance of the placenta. The immunological mechanism of tolerance of the semiallogenic fetus by the mother is still unclear despite much experimental work, as reviewed in ref. 17. Molecular studies of *HLA-G* gene-specific expression and transcriptional regulation might be helpful in order to elucidate this problem. In this work, we report an alternatively spliced form of the *HLA-G* transcript expressed in fetal trophoblasts and the presence of *HLA-G* mRNA in adult peripheral lymphocytes.

MATERIALS AND METHODS

Fetal Tissues and Adult Cells. Human first trimester trophoblasts were obtained from voluntary terminations of pregnancy at 6–10 weeks of gestation. Second trimester human fetal liver was obtained from therapeutic terminations of pregnancy at 16 weeks of gestation. Local ethical committee approval was obtained for this study. The tissues were washed extensively in PBS and a portion of them (trophoblasts or liver) were identified under the microscope; all materials were frozen immediately in liquid nitrogen. Samples of human peripheral blood were obtained from normal male volunteers. Mononuclear cells were separated from polynuclear cells by Ficoll/Hypaque density centrifugation and mRNA was isolated from both populations. Moreover, the mononuclear cells enriched in B lymphocytes were obtained by immunoabsorption on magnetic beads coated

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Abbreviations: MHC, major histocompatibility complex; RT, reverse transcriptase.

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with anti-CD19 antibody (Dynabeads; Dynal/Biosys, Compiègne, France) and those enriched in T lymphocytes were obtained by separation on Leuko-Pac (Fenwal Laboratories, Deerfield, IL). The enrichment was at ≈90% for B cells and ≈87% for T cells as estimated by fluorescence-activated cell sorting analysis using, respectively, fluorescein isothiocyanate-labeled anti-CD20 and anti-CD3 antibodies on 1×10^5 cells of each subpopulation.

RNA Isolation and Reverse Transcriptase (RT)-PCR Amplification. Total mRNA was isolated from 1 g of frozen tissue or 2×10^7 cells using RNA-Zol B reagent (Bioprobe Systems, Paris) according to the manufacturer's recommendations; the quality was verified by electrophoresis in denaturing 1.5% agarose gel. cDNA was prepared from 10 μg of total RNA with oligo(dT) priming and M-MLV RT (GIBCO/BRL; Life Technologies, Grand Island, NY) by incubation of a 20-μl mixture at 42°C for 1 h and then at 95°C for 5 min. *HLA-G*-specific primers were designed by comparison of classical and nonclassical *HLA* class I gene sequences using the BISANCE computer program (18); the sequences and relative positions of primers according to cDNA and genomic sequences are summarized in Table 1. The PCR fragments theoretically expected for *HLA-G*-specific primers, according to the full-length cDNA sequence, are listed in Fig. 1. To reduce the number of nonspecific amplimers PCR amplification was performed by using a hot-start technique (19). Briefly, in a first tube 200 μM each dNTP, 0.1 μg of each primer, and one pellet of AmpliWax (Cetus/Perkin–Elmer) in 50 μl of 1× PCR buffer was incubated at 75°C for 5 min; in a second tube 2 μl of RT reaction mixture or 1 μg of genomic DNA and 3.5 units of *Taq* polymerase (Cetus/Perkin–Elmer) in 50 μl of 1× PCR buffer was incubated at 95°C for 5 min. Then the contents of the tubes were mixed and subjected to 35 cycles of PCR under the following conditions: 94°C for 1 min, 61°C for 1 min, 72°C for 1 min 30 sec; the last extension step at 72°C was prolonged to 10 min. The PCR products were analyzed by electrophoresis in 1% agarose gel and stained with ethidium bromide. The specificity of PCR products was confirmed by alkaline blotting of the fragments in 0.4 M NaOH onto a nylon membrane (Hybond N^+; Amersham) and subsequent hybridization was performed in 5× SSPE/5× Denhardt's solution/0.5% SDS/100 μg of sonicated salmon sperm DNA per ml for 2 h at 55°C with ^{32}P-labeled oligonucleotide probe using [γ-^{32}P]dATP and a 5′-end-labeling kit (Boehringer Mannheim). In all PCR amplifications we used as controls the RT reaction mixture without M-MLV RT (RT^-) and the PCR mixture without the cDNA template (blank). Positive control of PCR amplifications was performed using HLA pan class I ubiquitous primers (Table 1).

Cloning and Sequencing of PCR Products. To generate templates for sequencing, the PCR fragments were separated by electrophoresis in a 4% polyacrylamide gel, cut out, eluted with 0.5 M ammonium acetate/5 mM EDTA, and reamplified by asymmetric PCR to generate single-strand products (20).

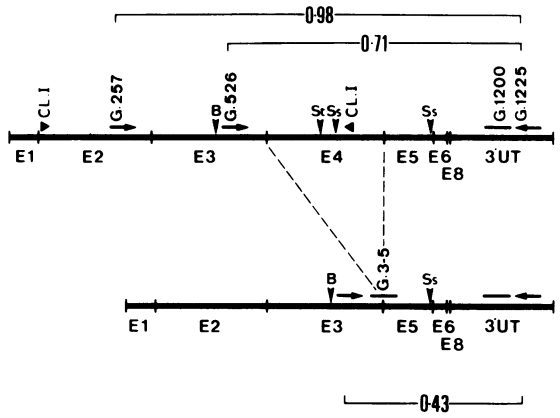

FIG. 1. Structure of *HLA-G* cDNA, locations of PCR primers (horizontal arrows), expected sizes (kb) of amplified fragments, and positions of *HLA-G*-specific probes used for hybridization (heavy bars). CL.I indicates HLA pan class I primers. Vertical arrowheads indicate restriction enzyme sites specific for exons 3, 4, and 5 useful for restriction analysis of RT-PCR products cloned in pPCRII vector. B, *Bgl* I; St, *Stu* I; Ss, *Sst* I.

The reamplified material was purified by phenol/chloroform extraction and twice precipitated with ethanol; then it was sequenced by using a T7 Sequenase 2.0 sequencing kit (United States Biochemical, Touzard-Matignon, France). In addition, the PCR products were cloned in pPCRII vector using a TA cloning system kit (Invitrogen) and then sequenced.

RESULTS

Detection of an Alternatively Spliced Transcript of the *HLA-G* Gene Lacking Exon 4 (*HLA-G.3–5*). PCR amplification was performed on cDNA from fetal first trimester trophoblasts and the products were analyzed by electrophoresis and hybridization. Using the primers *G.526–G.1225* two fragments at 0.71 and 0.43 kb were observed after gel electrophoresis and ethidium bromide staining, which hybridized with the *G.1200* probe (Fig. 2 *A* and *B*). As expected, the RT-PCR of mRNA from second trimester fetal liver does not show bands in gel and no hybridization signals were observed, even when amplification with HLA pan class I primers produced a positive signal (Fig. 2*C*). Amplification of genomic DNA produces one band at ≈2.2 kb according to the genomic *HLA-G* sequence. The larger fragment of 0.71 kb represents the full-length copy *HLA-G* transcript, whereas the fragment of 0.43 kb may correspond to a transcript that lacks the sequence encoded by 276 bp of exon 4 (*HLA-G.3–5*). To confirm that this fragment is generated from a transcript lacking exon 4, the 0.43-kb band was cut out and sequenced. As shown in Fig. 3, the 0.43-kb fragment sequence clearly presents a junction between exons 3 and 5 due

Table 1. Sequence and location of primers used for PCR and hybridization

Primer	Sequence (5′ → 3′)	Location	
		cDNA	Genomic
G.257 (+)	GGA AGA GGA GAC ACG GAA CA	257–276	Ex2
G.526 (+)	CCA ATG TGG CTG AAC AAA GG	526–545	Ex3
G.1200 (−)	CCC CTT TTC TGG AAC AGG AA	1200–1219	3′-UT
G.1225 (−)	TGA GAC AGA GAC GGA GAC AT	1225–1244	3′-UT
G.3–5 (+)	CAG CGC GCG GAG CAG TCT TC	615–624/901–910	Ex3/Ex5
Class I (+)	TCC CAC TCC ATG AGG TAT TTC	81–100	Ex2
Class I (−)	TCC AGA AGG CAC CAC CAC AG	814–833	Ex4

Ex, exon location according to ref. 5 for cDNA and according to ref. 13 for genomic DNA sequences; 3′-UT, 3′ untranslated region; G, HLA-G specific; Class I, HLA pan class I primer; (+) and (−), orientation of primer.

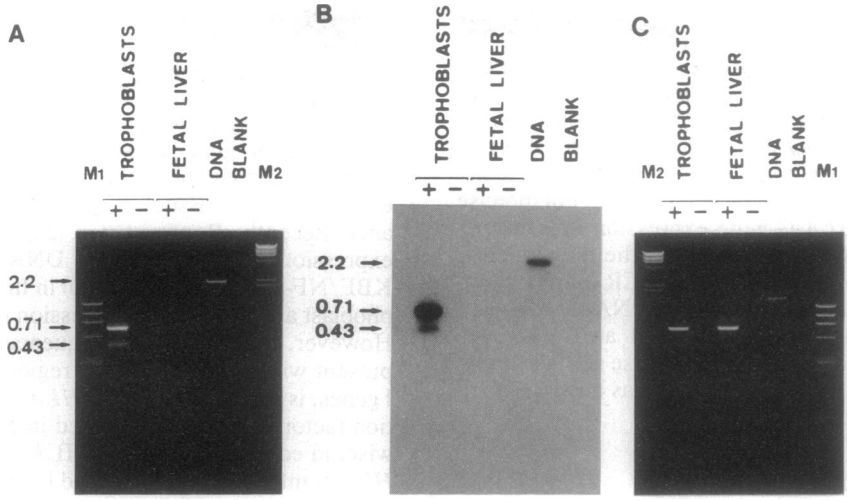

FIG. 2. Results of RT-PCR amplification obtained with *G.526–G.1225 HLA-G*-specific primers on fetal first trimester trophoblast cell cDNA and fetal second trimester liver cDNA analyzed by agarose gel electrophoresis (*A*) and hybridization with ^{32}P-labeled *G.1200* probe (*B*). Lanes + and − correspond to the RT$^+$ and RT$^-$ template. DNA lane corresponds to human genomic DNA amplification. (*C*) Amplification of cDNAs with HLA pan class I primers as positive control. Two bands at 0.71 and 0.43 kb are observed in stained agarose gel and hybridized Southern blot that are assigned, respectively, to full-length mRNA copy and alternatively spliced transcript lacking exon 4. M1 and M2, molecular size markers (ϕX174 RF DNA/*Hae* III and λ DNA/*Hin*dIII, respectively).

to the loss of exon 4. Moreover, sequencing of this alternatively spliced transcript reveals the absence of exon 7 and the presence of a stop codon in exon 6, as previously observed for other *HLA-G* mRNA forms (15); comparison to the published *HLA-G* sequences demonstrates homology with the sequence established by Shukla *et al.* (5) but different at base 1004 (A/G), 1123 (G/C), and 1165 (C/T).

Evaluation of Frequency of *HLA-G.3–5* Transcript. As deduced from Fig. 2*B*, the hybridization intensity of the 0.43-kb band corresponding to the spliced transcript is much weaker than that corresponding to the full-length copy; this suggests that the alternatively spliced transcript is less abundant in the *HLA-G* mRNA population. To evaluate relative amounts of these two transcripts, the PCR products from amplification of first trimester trophoblast mRNA with the primers *G.526–G.1225* were cloned in pPCRII vector. Of 260 clones analyzed by hybridization of replicas with *G.1200* or *G.3–5* probes, ≈210 clones showed a positive hybridization signal with *G.1200* and only one of them was positive with *G.3–5*. This unique clone was sequenced and the other five random *G.1200*-positive clones were identified by restriction analysis using the enzymes specific for exon 3 or 4 (Fig. 1). The sequence comparison demonstrated that the clone positive with the *G.3–5* probe lacked exon 4, whereas the five other clones corresponded to the full-length copy transcript (data not shown). Thus, the frequency of the alternatively spliced form vs. the full-length transcript may be estimated at ≈1/200.

Expression of *HLA-G* Transcript in Adult Peripheral Lymphocytes. Fig. 4 shows the results of PCR amplification obtained with *G.257–G.1225* primers on cDNA templates from peripheral mononuclear cells of one of six unrelated male adults tested. One band of 1.0 kb was observed in agarose gel (Fig. 4*A*) and subsequent hybridization with the *G.1200* probe revealed one band of identical size (Fig. 4*B*). According to the cDNA sequence, this band may correspond to the full-length *HLA-G* transcript. To confirm this, the PCR product was cloned in the pPCRII vector and then se-

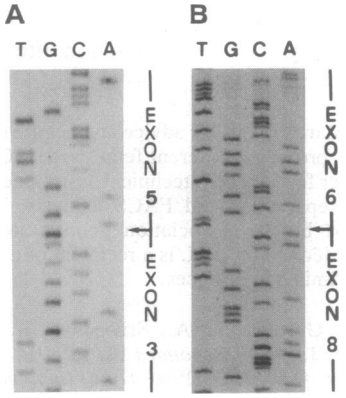

FIG. 3. Sequence analysis of PCR amplified 0.43-kb fragment from first trimester trophoblast cDNA. ^{35}S autoradiograms present the continuous sequence between exons 3 and 5 due to the lack of exon 4 (*A*) and the continuous sequence between exons 6 and 8 due to the lack of exon 7 (*B*).

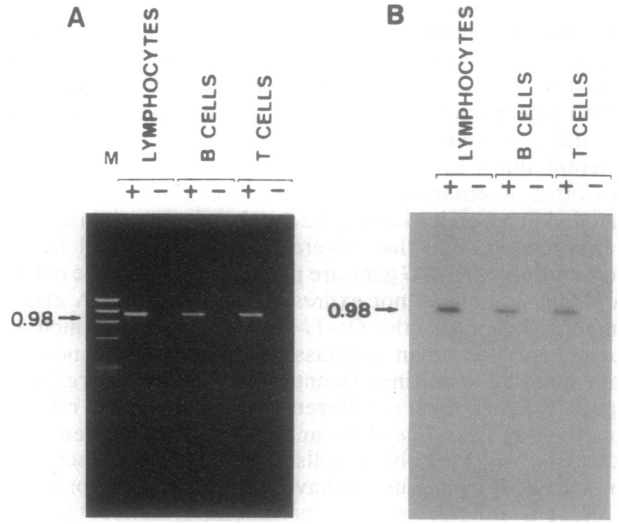

FIG. 4. RT-PCR amplification results obtained with primers *G.257–G.1225* on human adult peripheral lymphocytes and B- and T-cell fractions of cDNAs from one of six different donors analyzed by agarose gel electrophoresis (*A*) or hybridization with ^{32}P-labeled *G.1200* probe (*B*). One low intense band at 1.0 kb is observed in stained agarose gel and hybridized blot. This band is assigned to the full-length mRNA copy. M, molecular size marker (ϕX174 RF DNA/*Hae* III).

quenced. The 1.0-kb sequence of the PCR product is entirely homologous with the *HLA-G* sequence according to Shukla *et al.* (5). PCR amplification of cDNA from a polynuclear cell population with *HLA-G*-specific primers generates one very weak band of the same size (0.71 kb) that was observed in mononuclear cells, probably due to the presence of a few contaminant mononuclear cells in a polynuclear Ficoll/Hypaque separated fraction (data not shown). For precise cellular specificity of *HLA-G* gene expression in adult peripheral lymphocytes, we have separated the mononuclear cell populations and we carried out the PCR amplification with the *HLA-G*-specific primers on the cDNA from B- and T-cell-enriched subpopulations. One band at 1.0 kb was observed for B- and T-cell fractions in agarose gel as in a blot hybridized with *G.1200* probe (Fig. 4) and has been assigned to the full-length copy transcript.

DISCUSSION

We have demonstrated an alternatively spliced form of the *HLA-G* transcript in fetal trophoblasts that lacks exon 4, and we have shown evidence of the presence of the HLA-G mRNA in adult peripheral lymphocytes. It is well established that the *HLA-G* primary transcript is alternatively spliced in fetal placental tissues; two of them have already been identified by Ishitani and Geraghty (15). Nevertheless, the authors used the PCR primers encompassing exons 3 and 4 of *HLA-G* cDNA; thus, they could not detect the transcript lacking exon 4 by agarose gel electrophoresis separation or by hybridization of corresponding Southern blots because exons 3 and 4 have an identical length of 276 bp. However, by using an oligonucleotide primer specific for *HLA-G* exon 3 sequence (*G.526*) during PCR amplification, we obtained a positive selection of transcript lacking exon 4 rather than transcripts lacking exon 3 and exons 3 and 4. Thus, the 0.43-kb band observed in agarose gel as well as in a hybridized blot of PCR products corresponds specifically to mRNA lacking exon 4 as was also confirmed by sequencing. The frequency of this transcript appears low (≈1/200th of full-length copy mRNA). The lack of exon 4 created at the splice junction a GAG (Glu) codon rather than the GAC (Asp) codon found in the full-length transcript. Moreover, the lack of exon 4 excludes the $\alpha 3$ domain in the deduced protein sequence and confers a structure of HLA-G antigen that might be expressed on the surface of trophoblast cells. In this structure, the $\alpha 2$ domain and transmembrane region are bound together and may induce the conformational changes of surface protein; thus, the surface protein may present a different ability to bind to peptides. This may be true for the two other alternatively spliced *HLA-G* transcripts, which encoded the proteins lacking $\alpha 2$ or $\alpha 2$ and $\alpha 3$ domains. Thus, it may be possible that several isoforms encoded by the monomorphic *HLA-G* gene are present on the surface of fetal trophoblasts that do not express the classical HLA class I antigens. In mouse, the *Qa-T1* gene transcripts, which are homologous to human nonclassical HLA class I genes, are not expressed in murine placenta. Maybe *Qa-2*, corresponding to *HLA-G*, has a different function (7). In rat, the nonclassical class I gene *Pa* and classical class I genes are expressed on trophoblast cells (21). By using a sensitive hot-start PCR technique, we have demonstrated the presence of *HLA-G* mRNA in adult peripheral lymphocytes, this transcript being present in B- and T-cell subpopulations. The presence of *HLA-G* mRNA in lymphocytes is an intriguing finding with regard to the potential role of this gene in fetal–maternal tolerance; in fact, in these cells *HLA-G* transcript is present jointly with the classical HLA class I mRNAs. Nevertheless, on lymphocyte membranes only classical HLA class I antigens are expressed; thus, the regulation of *HLA-G* gene transcription and the role of the *HLA-G* transcript in peripheral lymphocytes should be investigated. In adult lymphocytes, we detected only one transcript that corresponds to full-length copy mRNA; further studies are necessary to determine the potential presence of the alternatively spliced forms of *HLA-G* mRNA.

A dominant goal of research focused on the *HLA-G* gene is understanding the mechanism of *HLA-G* expression in extraembryonic tissues in relation to fetal–maternal tolerance. Recently, Boucraut *et al.* (22) demonstrated the nuclear expression of the enhancer A DNA-binding proteins of the KBF/NF-κB/c-rel family (p50) in the first trimester cytotrophoblast and lack of this expression in syncytiotrophoblasts. However, the palindromic sequence related to enhancer A, present within the 5′-flanking region of classical HLA class I genes, is partially deleted in *HLA-G*, and another transcription factor is probably involved in *HLA-G* expression; likewise, in contrast to classical HLA class I, the expression of *HLA-G* mRNA is not increased by treatment with interferon γ, which might also be caused by 5′ end sequence modifications in the enhancer region (23). Otherwise, despite the presence of p50-related proteins in trophoblasts, the classical HLA class I genes are not expressed in this tissue, probably due to the methylated status of class I genes (24). By using transgenic mice, Schmidt *et al.* (25) located a positive regulatory region of human *HLA-G* within the 1.4-kb fragment at the 5′ end of the start site of transcription. Moreover, this fragment may contain additional regulatory elements that induce tissue-specific expression of the *HLA-G* gene in trophoblast cells of the placenta (26).

The immunological aspect of the fetal–maternal relationship also promises to be significant. Sanders *et al.* (27) have demonstrated that the T-lymphocyte differentiation marker CD8 can recognize and bind to HLA-G; thus, it is possible that HLA-G may serve as a recognition factor by $CD8^+$ cells (as suppressor T cells). Therefore, CD8 could be involved in recognition of HLA-G by $CD56^+$ cells as NK cells or uterine large granular lymphocytes—$CD2^+$, $CD3^-$, $CD56^+$ (28). Thus, HLA-G may protect the fetal trophoblasts from NK cell killing and confer immunological tolerance of fetus vs. mother. Another intriguing immunological problem concerns the finding that human fetal cells can cross the placental barrier and transfer into maternal circulation at about 8 weeks of gestation (29, 30) and may persist up to 8 months after delivery (31). It is unclear in which manner the fetal cells escape the mother's immunological control and possible cytotoxic reactions. Further investigations are necessary to correlate this with a possible expression of *HLA-G* gene products on cell membranes. Beyond the role the *HLA-G* gene products may play in fetal–maternal tolerance, the more relevant question is whether HLA-G may be a factor of protection against graft vs. host reaction after bone marrow grafts.

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