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Magali Le Discorde,2 Caroline Le Danff, Philippe Moreau, Nathalie Rouas-Freiss, and Edgardo D. Carosella

Service de Recherches en Hémato-Immunologie, CEA-DSV-DRM, Institut d’Hématoologie, Hôpital Saint-Louis, Paris 75010, France

ABSTRACT

Expression of the nonclassical HLA class I antigen, HLA-G, is associated with immune tolerance in view of its role in maintaining the fetus in utero, allowing tumor escape, and favoring graft acceptance. Expressed on invasive trophoblast cells, HLA-G molecules bind inhibitory receptors on maternal T lymphocytes and NK cells, thereby blocking their cytolytic activities and protecting the fetus from maternal immune system attack. The HLA-G gene consists of 15 alleles, including a null allele, HLA-G*0105N. HLA-G*0105N presents a single base deletion, preventing translation of both membrane-bound (HLA-G1) and full-length soluble isoforms (HLA-G5) as well as of the spliced HLA-G4 isoform. The identification of healthy subjects homozygous for this HLA-G null allele suggests that the HLA-G*0105N allele may generate other HLA-G isoforms, such as membrane-bound HLA-G2 and -G3 and the soluble HLA-G6 and -G7 proteins, which may substitute for HLA-G1 and -G5, thus assuming the immune tolerogenic function of HLA-G. To investigate this point, we cloned genomic HLA-G*0105N DNA and transfected HLA-G*0105N-transfected cells and were able to protect against NK cell lysis. These findings demonstrate that both membrane-bound and soluble HLA-G proteins are able to inhibit NK cell and antigen-specific T-cell cytotoxicity [3, 4] and the proliferation of allogeneic T cells [5–7]. Moreover, soluble HLA-G is able to induce apoptosis of both activated CD8+ T and NK cells [8, 9].

The identification of these seven HLA-G protein isoforms suggested that HLA-G provides multiple functions at the immune-privileged sites where they are expressed, such as the fetal-maternal interface, the thymus, and the cornea [10–12]. On the other hand, in the absence of a given HLA-G isoform, this redundancy may serve to maintain the immune tolerogenic function provided by one or more of the other HLA-G isoforms.

The HLA-G gene consists of 15 alleles, including the HLA-G*0105N null allele, which is characterized by a single base-pair deletion in exon 3 [13]. This deletion of a single cytosine at codon 130 results in a gap in the open reading frame, causing a premature stop at either codon 189 (TGA) near the beginning of exon 4, which blocks translation of HLA-G1 and -G5, or codon 297 (TAG) in exon 5, blocking the translation of HLA-G4. However, HLA-G*0105N is able to maintain translation of both the membrane-bound HLA-G2 and -G3 proteins and the soluble HLA-G6 and -G7 proteins, in all of which exon 3, containing the deletion, is removed by alternative splicing (Fig. 1).

The HLA-G*0105N null allele has been described in healthy adults whose own gestations and deliveries were normal (without complications). The detection of individuals who are genetically homozygous for the HLA-G*0105N allele suggests that the HLA-G isoforms encoded by this allele possess functions able to compensate for the absence of both the HLA-G1 and -G5 proteins and to maintain the immune privileged status of the fetal-maternal interface [14, 15].

Based on the previously mentioned observations and on a previous study showing that selection may have increased the frequency of the HLA-G*0105N allele [16], the goal of the present study was to demonstrate that in the absence of the HLA-G1, -G5, and -G4 isoforms, the other HLA-G isoforms present in HLA-G*0105N can indeed provide a protective function.

INTRODUCTION

Protection of the semiallogeneic fetus against maternal immune recognition and attack has been attributed principally to the high and almost unique expression of human leukocyte antigen-G (HLA-G) on trophoblast cells at the fetal-maternal interface [1]. The nonclassical HLA-G gene is characterized by alternative splicing that yields seven isoforms: four membrane (HLA-G1 through -G4) and three soluble (HLA-G5 through -G7). Full-length HLA-G mRNA encodes the HLA-G1 protein, which has three extracellular globular domains, one membrane-anchored domain, and one intracytoplasmic domain. Exons 3 and/or 4 may be deleted from the primary transcript, yielding the alternative mRNA HLA-G2, HLA-G3, and HLA-G4 forms. In addition, the insertion of introns 2 or 4 may generate soluble isoforms, such as HLA-G5 (the soluble full-length HLA-G1 counterpart), HLA-G6 (the soluble HLA-G2 counterpart), and HLA-G7 (the soluble HLA-G3 counterpart) (reviewed in [2]). The structures of both membrane-bound HLA-G1 and soluble HLA-G5 proteins are similar to those of classical class I proteins, consisting of three extracellular domains linked to β2-microglobulin (β2m).

Functional assays have demonstrated that both membrane-bound and soluble HLA-G proteins are able to inhibit NK cell and antigen-specific T-cell cytotoxicity [3, 4] and the proliferation of allogeneic T cells [5–7]. Moreover, soluble HLA-G is able to induce apoptosis of both activated CD8+ T and NK cells [8, 9].

The identification of these seven HLA-G protein isoforms suggested that HLA-G provides multiple functions at the immune-privileged sites where they are expressed, such as the fetal-maternal interface, the thymus, and the cornea [10–12]. On the other hand, in the absence of a given HLA-G isoform, this redundancy may serve to maintain the immune tolerogenic function provided by one or more of the other HLA-G isoforms.

The HLA-G gene consists of 15 alleles, including the HLA-G*0105N null allele, which is characterized by a single base-pair deletion in exon 3 [13]. This deletion of a single cytosine at codon 130 results in a gap in the open reading frame, causing a premature stop at either codon 189 (TGA) near the beginning of exon 4, which blocks translation of HLA-G1 and -G5, or codon 297 (TAG) in exon 5, blocking the translation of HLA-G4. However, HLA-G*0105N is able to maintain translation of both the membrane-bound HLA-G2 and -G3 proteins and the soluble HLA-G6 and -G7 proteins, in all of which exon 3, containing the deletion, is removed by alternative splicing (Fig. 1).

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In the present work, we investigate the structural and functional aspects of HLA-G*0105N proteins. We constructed the HLA-G*0105N null allele genomic DNA in the pcDNA3.1 expression vector, which enabled us to study the expression and function of its proteins. The aim of this work was to acquire more data on spliced HLA-G isoforms in general and to understand how they could compensate for the absence of full-length HLA-G proteins.

**MATERIALS AND METHODS**

**In Vitro Site-Directed Mutagenesis**

In vitro site-directed mutagenesis was carried out using the Stratagene QuickChange XL site-directed mutagenesis kit, which allows site-specific mutation in double-stranded plasmids. The plasmid DNA template (50 ng) HLA-G*0105N-pcDNA3.1, isolated from the dam1 JM109 Escherichia coli strain, was replicated with PhT Turbo DNA polymerase (2.5 U) using two synthetic primers, G.Null S and G.Null AS (125 ng each), consisting of 15 bases on both sides of the ΔC mutation. The oligonucleotide primers, each complementary to opposite strands of the HLA-G*0105N insert, were extended during temperature cycling (95°C for 1 min; 18 cycles at 95°C for 30 sec, 68°C for 15 min) and terminated by 7 min at 68°C. Following temperature cycling, amplification reactions were cooled and digested for 1 h at 37°C using Dpn I restriction enzyme to eliminate the nonmutated parental supercoiled double-stranded DNA. At this step, genomic HLA-G*0105N-pcDNA3.1 was replaced by genomic HLA-G*0105N-pcDNA3.1. The mutated DNA was then transformed in XL10-Gold ultracompotent cells.

**Cell Lines, Transfection, and Cultures**

Melanoma M8 (kindly provided by F. Jotereau, INSERM U211, Nantes, France) and histiocytic lymphoma U937 (American Type Culture Collection) human cell lines are HLA-class II negative; HLA-A, -B, -C, -G1, -G2, -G5, and -G6 isoforms made in our laboratory [25].

**Cell Surface Proteins**

The YT2C2 NK [19, 20] subclone (kindly provided by P. Paul, Hospital of Anthropology and Human Genetics, Munich, Germany) [17, 18] and collection human cell lines are HLA-class II negative; HLA-A, -B, -C, -G1, -G2, -G5, and -G6 isoforms made in our laboratory [25].

**Monoclonal Antibodies**

The following murine antibodies were used: 4H84, IgG1 recognizing α1 domain common to all HLA-G isoforms (provided by Michael Mc Master [23]); MEM-G09, IgG1 recognizing the denatured form of HLA-G1, -G2, -G5, and -G6 (provided by Vaclav Horejsi, Prague, Czech Republic); MEM-G09, IgG1 recognizing the HLA-G molecules associated with beta2-microglobulin (HLA-G1 and -G5) [24] (Exbio, Prague, Czech Republic); 5A6G7, IgG1 antibody against intron 4 of soluble HLA-G5 and -G6 isoforms made in our laboratory [25].

**Flow Cytometry Assays**

The flow cytometry technique was used to analyze cell-surface HLA-G protein expression. Transfected cells were phenotyped according to standard procedure by incubating them with the primary anti-HLA-G antibody MEM-G09 in PBS 2% heat-inactivated fetal calf serum for 30 min at 4°C. Cells were subsequently stained with an F(ab’)2 goat anti-mouse IgG antibody conjugated with phycoerythrin (Immunotech Coulter).
proteins were precipitated from the supernatant by precipitation with 100 μl of 50% streptavidin-agarose beads (Bio-Rad, Marnes la Coquette, France) for 1 h at 4°C. Nonbiotinylated proteins in the supernatant, the first centrifugation were taken to be the intracellular material and retained for Western blot analysis. Bead-bound biotinylated proteins were washed in three buffers (buffer 1: 0.1% CHAPS, 150 mM NaCl 40 mM, 0.05% NaN₃, and 20 mM Tris-HCl pH 7.5; buffer 2: 0.05% CHAPS, 0.1% SDS, 300 mM NaCl, and 10 mM Tris-HCl pH 8; buffer 3: 0.1% CHAPS and 20 mM Tris-HCl pH 7.4). Biotinylated proteins were then resuspended in 60 μl Laemmli buffer 2×, and 40 μl of the nonbiotinylated proteins were mixed with 20 μl Laemmli buffer 6×. These two samples were boiled for 5 min and analyzed by Western blot.

**Deglycosylation Treatment**

Forty microliters of M8 transfectants lysed in 0.5% CHAPS, 50 mM Tris-HCl pH 7.5 were deglycosylated, using peptide-N-glycosidase F from flavobacterium, according to Sigma’s instructions (Sigma-Aldrich). The deglycosylated proteins were examined by Western blot analysis.

**Western Blot Analysis**

Proteins were loaded and separated on 12% Tris-glycine-SDS polyacrylamide gels and electrophoblot to nitrocellulose membranes (Hybond, Amersham Biosciences, Orsay, France). The membranes were probed with an HLA-G-specific antibody (4H84, MEM-G/04), then revealed with peroxidase-conjugated sheep anti-mouse IgG Ab (Sigma-Aldrich). Western blots were developed by chemiluminescence (Amersham).

**Cytotoxicity Assays**

The cytolytic activity of the NKL cells and YT2C2 cells used as effectors (E) was assessed in 4-h 51Cr release assays in which the effector cells were mixed with 5 × 10⁵ 51Cr-labeled transfected-M8 or U937 (T) (100 μCi 51Cr sodium chromate; 1 Ci = 37 Gbq; Amersham) at various E:T ratios, as previously described [4]. The percentage specific lysis was calculated as follows: % specific lysis = [(cpm experimental − cpm spontaneous release)/(cpm maximum release − cpm spontaneous release)] × 100. Spontaneous release was determined by incubation of labeled target cells in RPMI 1640 medium supplemented with 10% FCS. Maximum release was determined by solubilizing target cells in 0.1 N HCl. In all experiments, spontaneous release was less than 10% of maximum release.

**RESULTS**

**Construction of HLA-G*0105N Null Allele**

**Genomic DNA**

To study proteins encoded by the HLA-G null allele, we generated a DNA sequence identical to HLA-G*0105N in the pCDNA 3.1 expression vector. In vitro site-directed mutagenesis of HLA-G*010102 genomic DNA allowed us to delete the cytosine at position 815 from ATG (exon 1), yielding the genomic DNA of the HLA-G*0105N allele. We used the HLA-G*010102 rather than the HLA-G*010101 reference sequence since HLA-G*010102 and -G*0105N have identical DNA sequences with the exception of the cysteine deletion at codon 130 [14]. The XbaI-EcoRV HLA-G*010102 fragment linked into the pCDNA3.1 vector used as the template is represented in Figure 1, and the electropherogram of DNA sequences before and after directed mutagenesis demonstrating deletion of the cysteine in the newly synthesized DNA is shown Figure 2, the remaining sequence being identical. The genomic HLA-G*0105N DNA presented a deletion at codon 130 (CTG → TGC), which generated a stop codon 171 nucleotides further on at codon 189 (GTA → TGA) in exon 4 or 495 nucleotides further on at codon 297 (GTA → TAG) in exon 5. At this step, we had obtained a copy of the HLA-G*0105N allele of 3932 nucleotides, beginning 460 bp upstream from exon 1 and ending 541 bp downstream from exon 8.

**Pattern of mRNA Expression in HLA-G*0105N-Transfected Cells**

Real-time RT-PCR was carried out on mRNA prepared from M8 cells transfected with both genomic HLA-G*0105N and HLA-G*010102 as well as mRNA from JEG-3 cells (Fig. 3). These results revealed that higher levels of HLA-G transcripts were produced in both M8-HLA-G*0105N and M8-HLA-G*010102 than in the JEG-3 cells (JEG-3 is taken as a reference since it is one of the rare cell lines that constitutively expresses HLA-G proteins). HLA-G transcript levels measured in the M8-transfected cells varied according to the particular HLA-G sequence.
transfected, lower levels being found for M8-HLA-G*0105N than for M8-HLA-G*010102 (Fig. 4). To determine the mRNA pattern of M8-HLA-G*0105N, we carried out HLA-G-specific RT-PCR and radioactive hybridization reactions comparing M8-HLA-G*0105N with M8-HLA-G*010102. The specific G.927 probe, located in exon 5, was used to hybridize all PCR products except those of G.257/G.14, which were hybridized using the G.R probe, located in exon 2. The G.257F/G.1004R primers disclosed four bands, corresponding to the principal mRNAs: HLA-G5 (889 bp), -G1 (767 bp), -G2 and -G4 (491 bp), and -G3 (215 bp) (Fig. 4a). HLA-G2 was amplified using the G.-3/G.1216 primers (627 bp) (Fig. 4b) and HLA-G3 with the G.-3/G.1216 primers (341 bp) (Fig. 4c). The G.257/G.14 primers allowed amplification of HLA-G5 (765 bp) and HLA-G6 (489 bp) (Fig. 4d). We thus confirmed that all HLA-G mRNA had been transcribed in M8-HLA-G*0105N cell line.

**HLA-G Proteins Are Translated in HLA-G*0105N-Transfected Cells**

The proteins synthesized by the HLA-G*0105N allele have been deduced from its nucleotide sequence [14]. According to these authors, HLA-G isoform amino-acid sequences deduced from the HLA-G*0105N DNA sequence would yield the complete membrane-bound HLA-G2 and -G3, as well as the soluble HLA-G6 and -G7 isoforms. HLA-G1, -G4, and -G5 undergo a reading frame shift, and the corresponding proteins are prematurely truncated in the null allele.

To detect HLA-G proteins that were expressed by M8-HLA-G*0105N in our experiments, we carried out immunocytochemistry assays on permeabilized cells. As may be seen in Figure 5, M8-HLA-G*0105N cells were positively stained by 4H84, an antibody that targets the α1 domain present in all HLA-G isoforms (Fig. 5b); by MEM-G/04, an antibody that recognizes the α3 domain (Fig. 5f); and by 5A6G7, an antisoluble antibody against HLA-G5 and -G6 (Fig. 5n). As expected, the presence of HLA-G1 or -G5 was not detected in M8-HLA-G*0105N with the MEM-G/09 antibody (Fig. 5j). All these anti-HLA-G antibodies had positively bound M8-HLA-G*010102, a genomic HLA-G sequence in which HLA-G1 is predominantly expressed, but one would expect the other isoforms to be present (Fig. 5, c, g, k, and o). The HLA-G2 isoform was stained by both the 4H84 and the MEM-G/04 antibodies in M8-HLA-G2 cells (Fig. 5, d and h). Collectively, these immunocytochemical analyses revealed the presence of HLA-G2 and -G6 isoforms in M8-HLA-G*0105N.

Using flow cytometry and the MEM-G/09 antibody, we confirmed that whereas M8-HLA-G*0105N did not express HLA-G1 protein at the cell surface, M8-HLA-G*010102 did (Fig. 6). Because of the unavailability of antibodies able to bind HLA-G2 and HLA-G3 in the flow cytometry assay, we were unable to identify these membrane-bound isoforms, which could have been translated by M8-HLA-G*0105N cells.

**Characterization of HLA-G Proteins in HLA-G*0105N-Transfected Cells**

In succeeding experiments, we attempted to characterize the HLA-G proteins that had been translated in M8-HLA-G*0105N cells, using M8-HLA-G*010102 and M8-HLA-G2 as positive controls. We used immunoprecipitation of biotinylated cell-surface proteins to achieve separation of membrane-bound and intracytoplasmic proteins. The results revealed membrane-bound HLA-G2 protein on M8-HLA-G2 (bands at 30 and 27 kDa) and HLA-G1 protein on M8-HLA-G*010102 (one band at 43 kDa), but no HLA-G molecules could be detected in M8-HLA-G*0105N by this method (Fig. 7A). However, study of the intracellular pro-
proteins revealed the presence of HLA-G isoforms. We obtained three bands, one at 30 kDa and one at 27 kDa, that were identical to those produced by M8-\(\text{HLA-G}^*\)2, plus one located above them, at 35 kDa, that has never been characterized (Fig. 7B). To determine whether this latter band corresponded to hyperglycosylated HLA-G2, we treated the M8-\(\text{HLA-G}^*\)0105N cells with PNGase F, an enzyme that catalyzes the removal of N-linked oligosaccharide chains from glycoproteins (Fig. 8). This enzyme treatment revealed a smaller band just below that of the mature protein, corresponding to the deglycosylated protein. This finding indicated that M8-\(\text{HLA-G}^*\)0105N had indeed translated some glycoproteins, but the molecular weight obtained for the 35-kDa protein did not correspond to any known HLA-G isoform. Staining with 4H84 and MEM-G/04 antibodies allowed us to determine that at least the \(\alpha 1\) and \(\alpha 3\) domains were present in this protein, which remains to be characterized.
from the nucleotide sequence of G*0105N used site-directed mutagenesis to construct the HLA- proteins generated by the to identify the structural and functional characteristics of maternal-fetal tolerance \[27, 28\], we designed experiments against M8- by binding to HLA-G molecules. The lytic activity of NKL which are implicated in the NK-cell cytotoxicity pathways, While YT2C2 express only KIR2DL4, NKL cells express similar proliferative responses and natural killing function. most of the original features of NK cells, including very with interleukin-2, the NKL human NK cell line retained efectant cells used as the target (Fig. 9). When stimulated with interleukin-2, the NKL human NK cell line retained most of the original features of NK cells, including very similar proliferative responses and natural killing function. While YT2C2 express only KIR2DL4, NKL cells express both ILT2 and KIR2DL4 inhibitory receptors \[18, 26\], which are implicated in the NK-cell cytotoxicity pathways, by binding to HLA-G molecules. The lytic activity of NKL against M8-HLA-G*0105N was compared with its lytic ac- tivity against M8-HLA-G*010102 cells, which at least express HLA-G1, as well as against M8-HLA-G2 cells, which express only the HLA-G2 isoform, and against M8-pcDNA, used as the HLA-G-negative control. Cytotoxicity experi- ments showed that in addition to M8-HLA-G2, both geno- mic M8-HLA-G*0105N and M8-HLA-G*010102 were pro- tected from NKL lysis. Lysis of M8-transfectants was re- duced by 50% compared with that of the M8-pcDNA control cell line. Inhibition of cytotoxicity was also obtained using YT2C2 cells, which only express the HLA-G-specific receptor against U937-transfectants.

**DISCUSSION**

After our study of the tolerogeneic role of HLA-G in maternal-fetal tolerance \[27, 28\], we designed experiments to identify the structural and functional characteristics of proteins generated by the HLA-G*0105N allele. First, we used site-directed mutagenesis to construct the HLA-G*0105N from the nucleotide sequence of HLA-G*010102 and transfected it into the M8 human cell line. Then we characterized the transcripts and proteins generated by the HLA-G*0105N gene before analyzing the functional capacity of the isoforms produced.

Although the deletion of one base in exon 3 of HLA- G*0105N disrupts the reading frame, it should have no ef- fect on transcription of the primary transcript or on its al- ternative splicing \[14\]. Indeed, in our RT-PCR analysis, the HLA-G mRNA profile was the same in both M8-HLA- G*0105N- and M8-HLA-G*010102-transfected cells, indic- ating the presence of all HLA-G mRNA transcripts nor- mally found. Thus, deletion of the cytosine in codon 130 did not block the transcription and splicing mechanisms in the null allele. Nevertheless, real-time and classical RT- PCR showed that HLA-G mRNA levels were lower in M8-HLA-G*0105N than in M8-HLA-G*010102 cells, and classical PCR revealed that the HLA-G1/HLA-G2 mRNA ratio was lower in M8-HLA-G*0105N than in M8-HLA- G*010102. This discrepancy in the expression level of HLA-G genotypes and isoforms has already been reported for alleles other than the null allele in pathological preg- nancies in which altered HLA-G transcription was associ- ated with certain HLA-G genotypes \[29, 30\] as well as in a study of the role of HLA-G alleles in determining the soluble HLA-G protein plasma level \[31\]. Another expla- nation is that nonsense-mediated decay, a eukaryotic regul- atory process that degrades mRNA with premature termi- nation codons, could be responsible for the higher deg- radation of HLA-G1 transcripts in cells expressing the null allele compared with other alleles \[32\].

Furthermore, the higher levels of mRNA in transfected cells compared with JEG-3 cells could be explained by the fact that 1) in transfected cells, HLA-G is ligated in the pcDNA3.1 plasmid, which possesses the CMV promoter and is thus able to influence and perhaps increase HLA-G mRNA expression, and 2) HLA-G*010102 has been described as a much higher “HLA-G secreting” allele than either HLA-G*0105N or HLA-G*010103 (carried by JEG-3 cells \[33\]) \[31\].

Finally, although mRNA levels are higher in M8-trans- fected cells than in JEG-3 cells, this cannot be correlated with either the quality or the quantity of the corresponding
FIG. 9. M8-HLA-G*0105N is able to inhibit NKL cell cytolytic activity. Chromium release assays were carried out using M8- or U937-pcDNA, M8- or U937-HLA-G*0105N, M8- or U937-HLA-G*010102, or M8-HLA-G2 as target cells and NKL cell line (A) or YT2C2 cell line (B) as effectors. Results are expressed as the percentage lysis recorded in a 4-h $^{51}$Cr-release assay. Values represent means of triplicate ± SD. This experiment is representative of five distinct experiments.

translated isoform proteins. Thus, the mRNA level is lower in M8-HLA-G*0105N cells than in M8-HLA-G010102 cells (Fig. 3), and both M8 transfectants are equally protected from NKL-mediated cytolysis (Fig. 9A).

Since the presence of HLA-G mRNA had been demonstrated in M8-HLA-G*0105N, we also looked for HLA-G proteins in it. For this purpose, HLA-G*0105N-transfected cells were immunostained with specific HLA-G antibodies targeting either all HLA-G proteins or specific isoforms. As expected from the deletion in HLA-G*0105N, neither the HLA-G1 nor the HLA-G5 isoform was detected in M8-HLA-G*0105N since these cells were not stained by the MEM-G/09 mAb, which is specific for HLA-G1 and HLA-G5. The absence of HLA-G5 and of shed HLA-G1 was confirmed by a specific ELISA (data not shown). Interestingly, the other mAbs that recognized either all HLA-G isoforms (4H84), both HLA-G5 and -G6 (5A6G7), or HLA-G1, -G2, -G5, and -G6 (MEM-G/04) were positive in M8-HLA-G*0105N, demonstrating that in the absence of both HLA-G1 and -G5, other HLA-G isoforms are produced. These isoforms may correspond to at least the HLA-G2 and/or HLA-G6 proteins. In support of this observation, previous immunohistochemical analyses of placentas homozygous for HLA-G*0105N presented negative staining with an anti-HLA-G1, -G5 antibody (87G) and positive staining with an anti-HLA-G1, -G2 antibody (15C6) [15]. These results confirm that HLA-G proteins are indeed translated in HLA-G*0105N cells.

To further characterize the HLA-G isoforms produced by M8-HLA-G*0105N cells, we carried out immunoprecipitation and Western blot experiments. Under our experimental conditions, proteins at 35 and 30 kDa were detected within such cells but not on the cell surface. However, we cannot exclude that HLA-G proteins are secreted by these cells and that they exercise biological effects. To address this point, we carried out experiments aimed at analyzing the role of the HLA-G proteins generated by M8-HLA-G*0105N with respect to NK function and tested their capacity to inhibit NK cell lysis.

Considered together, these results demonstrate that non-HLA-G1/-G5 isoforms of the HLA-G*0105N allele participate in the inhibition of NK cells. This inhibition is perhaps not mediated by HLA-E or other HLA class I molecules, since HLA-G*0105N-transfected cells were protected from lysis by the NK-like clone Y2T2C2. Indeed Y2T2C2 expresses the HLA-G-specific receptor KIR2DL4, but not HLA-A-, -B-, -C-, and -E-specific KIRs.

The HLA-G*0105N allele is estimated to have appeared 18 000 yr ago, leading to the conclusion that it had been positively selected during evolutionary history [16]. A high incidence of the null allele has notably been reported in Africa [34]. A possible explanation for this selection is that it may be due to the high incidence of intrauterine pathogens among the Africa population, which therefore has to maintain an efficient immune system to eliminate uterine infections [16]. One would expect that the absence of HLA-G1 and -G5 in HLA-G*0105N individuals would serve to maintain the immunocompetence of maternal NK and T cells at the maternal-fetal interface [15, 16]. Weighing the trade-off between increased risk of miscarriage due to the
absence of HLA-G1 and -G5 but low risk of uterine infection and successful outcome of pregnancy but high risk of womb infection, HLA-\textit{G*0105N} was selected over HLA-G1, -G5, thus eradicating intrauterine pathogen contamination despite the fact that the null genotype is a contributing factor in spontaneous abortion [35, 36]. Our results suggest that HLA-\textit{G*0105N} would be of interest for purposes other than merely preventing interactions between HLA-G1, -G5 and inhibitory NK receptors since HLA-G2 has maintained that function. The advantages of the selection of this allele must be further investigated.

The emergence of the HLA-\textit{G*0105N} homozygous gene is not the only condition under which HLA-G protein expression has been found to be impaired. As for classical HLA-class I molecules, both HLA-G1 and -G5 contain three globular domains associated with \(\beta_2m\) and present peptides. Their conformation renders these HLA molecules TAP and \(\beta_2m\) dependent. Therefore, in TAP- or \(\beta_2m\)-deficient individuals, NK-mediated reactions may still occur, and NK cells may retain the ability to reject allogeneic and autologous HLA-class I-negative cells. However, studies of such individuals showed that HLA-class I-deficient cells remain protected from NK lysis. Notably, there are other situations in which expression of both HLA-G1 and -G5 isoforms would also be altered without negatively affecting the outcome of pregnancy. One example is the survival of homozygous TAP-negative children in whom one would expect HLA-G1/G5 placental expression to be impaired. Indeed, expression of these two isoforms as a trimolecular \(\alpha\)-chain/\(\beta_2m\)/peptide complex is TAP dependent. In the fetuses of such TAP-negative children, the other HLA-G isoforms, whose expression may be independent of peptide loading via TAP, may substitute for the loss of HLA-G1/G5, thus contributing to the survival of these fetuses [37].

In another study, functional analyses indicated that TAP-/- NK cells were unable to kill autologous class I-negative cells. This inhibition was attributed to an unknown inhibitory receptor capable of binding ligand expressed by targeted autologous class I-negative cells, thereby down-regulating NK cell cytotoxicity [38]. Considered together, these studies suggest that still-unknown mechanisms prevent an attack against autologous normal cells that express insufficient quantities of HLA-class I molecules. Therefore, the HLA-G2, -G3, -G6, and -G7 isoforms, in which cell processing and transport are probably peptide independent, might be candidate molecules to compensate for the loss of HLA class I antigens, including HLA-G1 and -G5. The fact that TAP-/- NK cells express an unidentified receptor suggests that this unidentified inhibitory receptor is specific for spliced HLA-G isoforms. These interpretations could be extrapolated to tumor cells, which often present down-regulation of classical and nonclassical HLA class I molecules. Expression of the TAP and \(\beta_2m\)-independent spliced HLA-G isoforms (HLA-G2 through -G7) observed in some tumors may constitute a new escape mechanism from NK cell-mediated lysis [39].

Finally, our study provided information indicating that HLA-\textit{G*0105N} does not encode the complete HLA-G1 or HLA-G5 isoforms but does encode functional HLA-G proteins able to inhibit NK cell cytosis. Thus, although the biological functions of HLA-G1 and HLA-G5 proteins are abrogated, the other isoforms can assume their roles. Two different effectors were used against M8-transfectant cells: the NKL and YT2C2 cells. Since NKL cells expressed ILT2, KIR2DL4, and CD94/NKG2A receptors, lysis of HLA-\textit{G*0105N} cells could be due to a direct interaction between HLA-G and ILT2 or KIR2DL4 receptors and/or to a indirect effect through HLA-E (whose surface expression is up-regulated in presence of HLA-G leader peptide) and CD94/NKG2A. Nevertheless, results obtained with the YT2C2 effector cells demonstrate that at least HLA-G proteins expressed by HLA-\textit{G*0105N} inhibit NK lysis through direct interaction with KIR2DL4. Our results showing that HLA-\textit{G*0105N} cells retained the capacity to inhibit NK cytotoxicity activity agree with the previously described inhibitory role played by the HLA-G2 and -G3 [4]. In support of a biological role for other HLA-G isoforms than -G1 and -G5, two recent reports found that 1) HLA-G2 and -G6 isoforms are expressed by specific subpopulations of trophoblast cells [40] and that 2) recombinant HLA-G6 induces TGF-\(\beta\) production by monocyctic lineage cells [41].

Most classical HLA-class I genes are translated into one isoform. Therefore, a mutation in the gene yields a null allele, which results in the disappearance of this HLA molecule. A feature of the HLA-G gene is that it encodes seven isoforms (HLA-G2 through -G7) observed in some tumors, whose expression may be independent of peptide availability. Thus, although the expectation of HLA-G1/-G5 placental expression to be impaired, the emergence of the HLA-\textit{G*0105N} allele, which results in the disappearance of this HLA molecule, is not the only condition under which HLA-G protein expression has been found to be impaired. As for classical HLA-class I molecules, both HLA-G1 and -G5 contain three globular domains associated with \(\beta_2m\) and present peptides. Their conformation renders these HLA molecules TAP and \(\beta_2m\) dependent. Therefore, in TAP- or \(\beta_2m\)-deficient individuals, NK-mediated reactions may still occur, and NK cells may retain the ability to reject allogeneic and autologous HLA-class I-negative cells. However, studies of such individuals showed that HLA-class I-deficient cells remain protected from NK lysis. Notably, there are other situations in which expression of both HLA-G1 and -G5 isoforms would also be altered without negatively affecting the outcome of pregnancy. One example is the survival of homozygous TAP-negative children in whom one would expect HLA-G1/G5 placental expression to be impaired. Indeed, expression of these two isoforms as a trimeric \(\alpha\)-chain/\(\beta_2m\)/peptide complex is TAP dependent. In the fetuses of such TAP-negative children, the other HLA-G isoforms, whose expression may be independent of peptide loading via TAP, may substitute for the loss of HLA-G1/G5, thus contributing to the survival of these fetuses [37].

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### REFERENCES


23. McMaster M, Zhou Y, Shorter S, Kapasi K, Geraghty D, Lim KH,

22. Riteau B, Moreau P, Menier C, Khalil-Daher I, Khosrotehrani K, Bras-

20. Riteau B, Menier C, Khalil-Daher I, Martinozzi S, Pla M, Dausset J,


disequilibrium and age estimates of a deletion polymorphism
(1597DeltaC) in HLA-G suggest non-neutral evolution. Hum Immunol


12. Le Discorde M, Moreau P, Sabatier P, Legeais JM, Carosella ED.
Expression of HLA-G in human cornea, an immune-privileged tissue.

11. Le Discorde M, Moreau P, Cabestre FA, Menier C, Khalil-
P, Villena AA, Giacomini P, Natali PG, Guamini F, Ferrara GB,
McMaster M, Fisher S, Schust D, Perrone S, Duessert J, Geraghty D,
Carosella ED. HLA-G, -E, -F co-expression boosts the HLA class I-mediated NK lysis inhibition. Int Immunol 2001; 13:193–


disequilibrium and age estimates of a deletion polymorphism
(1597DeltaC) in HLA-G suggest non-neutral evolution. Hum Immunol

15. Robertson MJ, Cochran KJ, Cameron C, Le JM, Tantravahi R, Ritz


12. Le Discorde M, Moreau P, Sabatier P, Legeais JM, Carosella ED.
Expression of HLA-G in human cornea, an immune-privileged tissue.

11. Le Discorde M, Moreau P, Cabestre FA, Menier C, Khalil-
P, Villena AA, Giacomini P, Natali PG, Guamini F, Ferrara GB,
McMaster M, Fisher S, Schust D, Perrone S, Duessert J, Geraghty D,
Carosella ED. HLA-G, -E, -F co-expression boosts the HLA class I-mediated NK lysis inhibition. Int Immunol 2001; 13:193–


disequilibrium and age estimates of a deletion polymorphism
(1597DeltaC) in HLA-G suggest non-neutral evolution. Hum Immunol

15. Robertson MJ, Cochran KJ, Cameron C, Le JM, Tantravahi R, Ritz