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HLA-G Isoforms Produced by Placental Cytotrophoblasts and Found in Amniotic Fluid Are Due to Unusual Glycosylation

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The human placenta expresses HLA-G, a nonclassical (class Ib) MHC molecule that could play a central role in maternal tolerance of the semiallogeneic fetus. In this work, we report the production of a new mAb, 4H84, that specifically reacts with HLA-G in two formats: immunochemistry and immunoblotting. Immunolocalization experiments with 4H84 confirmed our previous finding that cytotrophoblasts within the uterine wall are the only cells in tissue sections of placenta that express the HLA-G protein. Additional experiments showed that both amniocytes and cytotrophoblasts in the amnion-chorion express this protein. Since multiple HLA-G transcripts have been described, we used immunoblotting to study the HLA-G isoforms produced by cytotrophoblasts in vitro and by the amnion-chorion in vivo. Cytotrophoblasts, their conditioned medium, and amniotic fluid samples contained heterodisperse immunoreactive bands (M_r 35,000–50,000). N-deglycosylation by peptide-N-glycosidase F digestion resolved these isoforms into two distinct bands. Cell samples contained primarily an M_r 37,000–42,000 protein, most likely encoded by the full-length mRNA. Conditioned medium and amniotic fluid contained a slightly smaller protein, most likely the secreted form lacking the transmembrane and cytoplasmic regions. Removal of polylactosamine chains by endo-β-N-acetyllactosaminidase digestion significantly reduced the electrophoretic mobility of the immunoreactive bands, suggesting that HLA-G, unlike class Ib molecules studied to date, carries N-acetyllactosamine units. These data show that M_r heterogeneity of HLA-G is due to its novel glycosylation, rather than to the translation of alternatively spliced mRNAs. We postulate that the unusual carbohydrate structures this molecule carries could interact with maternal immune cells and/or stabilize the molecule. The Journal of Immunology, 1998, 160: 5922–5928.

Since one-half of the fetal genes are derived from the father, the conceptus has the potential to stimulate maternal immune reactivity against paternally encoded Ags, including histocompatibility molecules. Why the semiallogeneic feto-placental unit does not evoke a maternal immune response is largely unknown. Trophoblasts, the specialized epithelial cells of the placenta, have been studied intensely in the context of maternal tolerance because they form the interface between the maternal and fetal compartments.

Early in human pregnancy, cytotrophoblast stem cells differentiate into distinct trophoblast populations that ultimately reside in different placental compartments; consequently, their interactions with maternal cells also differ (reviewed in Refs. 1 and 2). In one differentiation pathway, the cytotrophoblast stem cells fuse to form a multinucleate syncytiotrophoblast. These syncytiotrophoblasts cover the surface of floating chorionic villi, which are bathed in maternal blood. Their location is ideally suited to their function: gas, nutrient, and waste exchange. In another differentiation pathway, the stem cells differentiate into tumor-like cells that leave anchoring chorionic villi to aggressively invade the uterine wall and its vasculature. This subpopulation of extravillous cytotrophoblasts anchors the conceptus to the uterus and establishes the flow of maternal blood to the placenta. In this location, they come into direct contact with several types of maternal cells, including a unique population of NK cells that are abundant in the pregnant uterus (3, 4). Another subpopulation of extravillous cytotrophoblasts lies adjacent to the amniotic epithelium. Collectively, these chorionic cytotrophoblasts, the amniocytes, and the connective tissue with which they are associated are termed the amnion-chorion.

HLAs present peptides to immune cells and are, therefore, important determinants of alloreactivity. Thus, defining the trophoblast HLA repertoire is central to understanding the role of the placenta in immune interactions with maternal cells. None of the trophoblast populations expresses HLA-A, HLA-B, or class II (HLA-D) MHC molecules. Whether these cells express HLA-C is controversial (5, 6). But it is well established that cytotrophoblasts that invade the uterus express the full-length nonclassical (class Ib) HLA protein, HLA-G (7–11), and secrete a truncated form of this molecule (8). Although HLA-G mRNA has been detected in other adult and fetal tissues by reverse-transcriptase PCR analyses (for a review, see Ref. 12), a subpopulation of thymic epithelia are the only other cells that are known to express the protein (13).

Like the extensively studied class Ia genes, HLA-G contains eight exons that encode three extracellular domains, a transmembrane region, and a cytoplasmic tail. The single N-linked glycosylation site (Asn^93) present in all class I molecules is conserved, as are the structurally important cysteines in the α2 and α3 domains (14). However, studies at the mRNA level suggest a high degree of complexity. To date, six different alternatively spliced HLA-G mRNAs have been reported. In addition to the full-length...
(G1) form, transcripts lacking exon 3 (G2), exons 3 and 4 (G3), or exon 4 (G4) have been described (15, 16). These mRNAs encode proteins that lack either the α2, α2 and α3, or α3 domains. Additionally, cDNAs that potentially encode soluble molecules have been reported (17, 18). Thus, protein products of the HLA-G gene could vary widely in both m.w. and function.

Determining which of these mRNAs is translated into protein has been difficult due to the lack of suitable reagents. In this work, we report the generation of mAbs that detect HLA-G via an α1 domain epitope that is present in all of the potential forms of HLA-G proteins described to date. We used this Ab to study HLA-G expression in the placenta, amnion-chorion, and amniotic fluid. In tissue sections, the Ab reacted with invasive cytotrophoblasts, chorionic cytotrophoblasts, and amniocytes. Immunoblot analyses showed heterodisperse bands (M_r 35,000–50,000) that resolved into two major species after enzymatic N-deglycosylation. Cell samples contained primarily an M_r 37,000–42,000 band, most likely the plasma membrane form of the protein. Conditioned medium and amniotic fluid contained a slightly smaller protein, most likely the secreted form. These results suggest that the m.w. heterogeneity of HLA-G observed at the protein level is due to an unusual type of glycosylation, rather than to the translation of alternatively spliced mRNAs.

Materials and Methods

mAb production

The initial stages of mAb production were performed essentially as described (11). Briefly, BALB/c mice (Charles River, Wilmington, MA) were immunized with a peptide corresponding to amino acids 61 to 83 of the α1 domain of HLA-G (EEETNKTKAHTDRMLNLQTLRG) coupled to keyhole limpet hemocyanin. Splenocytes were fused according to standard methods. Hybridomas that secreted Abs reactive with the peptide immunogen were identified by ELISA.

In this study, lines that produced anti-peptide Abs were tested in a secondary screen for their ability to react with the HLA-G heavy chain in cytotrophoblasts and JEG-3 cells lysates by immuno blotting. Those that did were tested in a tertiary screen for their ability to react with invasive cytotrophoblasts in tissue sections of the maternal-fetal interface, as previously described (11). Several promising lines were chosen and cloned by secondary screen for their ability to react with the HLA-G heavy chain in cytotrophoblasts, chorionic cytotrophoblasts, and amniocytes. Conditioned medium and amniotic fluid contained a slightly smaller protein, most likely the secreted form. These results suggest that the m.w. heterogeneity of HLA-G observed at the protein level is due to an unusual type of glycosylation, rather than to the translation of alternatively spliced mRNAs.

Tissue and amniotic fluid collection

Informed consent was obtained from all patients from whom tissue, fluid, and blood samples were collected. Placentas and amnion-chorion were obtained from elective pregnancy terminations. Lekucocytes were isolated from blood by centrifugation through Ficoll-Hypaque 1027 (Sigma, St. Louis, MO).

Amniotic fluid samples collected during weeks 16 to 18 of pregnancy were obtained from the UCSF Cytogenetics Laboratory (San Francisco, CA). Amniotic fluid samples collected during the third trimester of pregnancy were obtained after amniocentesis for fetal lung maturity assessment. Fluids were stored at 4°C before centrifugation to remove cells, then stored at −20°C until analyzed.

Cervicovaginal fluid samples were obtained from women in labor at term. Cotton swabs were used to collect fluid samples from the cervical os and posterior fornix of women undergoing speculum examination for assessment of membrane integrity and the progress of labor. The status of their membranes was then determined using the ferning and nitrazine tests. The swabs were stored at −20°C until extraction with SDS-PAGE sample buffer, then analyzed by immunoblot.

Immunofluorescence

Immunofluorescence was performed as previously described on frozen sections of either first trimester placental bed samples or second trimester amnion-chorion (11). Hybridoma 4H84-conditioned medium was diluted 1/100. All sections were double stained with 4H84 and anti-cytokeratin (7D3).

Preparation of cell lysate and conditioned medium samples

Highly purified cytotrophoblasts were isolated from first, second, and third trimester chorionic villi, as previously described (20, 21). Cells (1 × 10^6) were plated in 35-mm culture wells coated with the extracellular matrix preparation Matrigel (Collaborative Research, Bedford, MA) in 2 ml MEM (UCSF Cell Culture Facility) containing 2% Nutridoma (Boehringer Mannheim, Indianapolis, IN). After 36 to 48 h, the conditioned medium was centrifuged to remove any cellular debris, aliquoted, and frozen at −80°C until analysis. The cells were collected in cold lysis buffer containing 150 mM NaCl, 10 mM Tris (pH 8), 0.5% Nonidet P-40 (Sigma), and protease inhibitors (1 mM PMSF, 5 mM EDTA, and 5 µg/ml apro tin). Lysates were clarified by centrifugation at 16,000 × g for 15 min at 4°C.

Anchoring chorionic villi were dissected from placentas and cultured for 12 to 36 h, as previously described (22), before the cells and conditioned medium were processed as described for cytotrophoblasts. JEG-3 cells were cultured in MEM (UCSF Cell Culture Facility) supplemented with 10% FBS (HyClone, Logan, UT) and processed as for the cyt trophoblasts and their conditioned medium. PBL lysates were also prepared as described above for the cytotrophoblasts.

Immunoblotting

Samples were resolved in 10% SDS-PAGE gels and electroblotted to nitrocellulose membranes (Schleicher & Schuell, Keene, NH), according to published methods (23). Membranes were blocked in PBS containing 0.1% Tween-20 (Sigma) and 5% Carnation nonfat dry milk (T-blotto) for 1 h at room temperature, and then incubated with mAb 4H84 (hybridoma supernatant diluted 1/100) in T-blotto) for 1 h at room temperature. Next, membranes were washed (3 × 10 min) with PBS-Tween and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson Immuno Research, West Grove, PA) diluted 1/2000 in T-blotto for 30 min at room temperature. Membranes were then processed for chemiluminescence according to the manufacturer’s instructions (ECL; Amersham, Buckinghamshire, England), and exposed to autoradiography film (Hyperfilm; Amersham).

Glycosidases treatments

Pep tide-N-glycosidase F (peptide N4-[N-acetyl-β-galactosaminyl] asparagine amidase F; PNGase F) was obtained from Boehringer Mannheim. Before N-deglycosylation, cell lysates, conditioned media, amniotic fluid, and cervical swab samples were boiled for 5 min in 20 mM NaPO_4 (pH 7.2, 50 mM EDTA, 10 mM sodium azide, and 0.05% SDS). PNGase F was then added (8 U/ml), and the samples were incubated overnight at 37°C. Endog enous β-D-galactosidase was obtained from V-Labs (Covington, LA). Digestions with this enzyme (overnight at 37°C) were conducted in 50 mM sodium acetate, pH 5.5. Following glycosidase treatment, samples were boiled in SDS-PAGE sample buffer and either electrophoresed immediately or stored at −80°C for later analyses.

Results

Production of anti-HLA-G mAbs and their use in immunolocalization studies

In a previous study, we used Abs directed against a peptide that corresponds to amino acids 61–83 of the HLA-G α1 domain to show, by immunolocalization on tissue sections, that invasive cytotrophoblasts express this 1b molecule in vivo (11). Since it was not possible to use these IgM mAbs for immunoblotting, we used the same immunogen to produce a second generation of anti-HLA-G (IgG) mAbs that could be used for both immunochemistry and immunoblotting. Several clones from this fusion had the desired properties. We routinely used an IgG1 mAb produced by one of these clones (4H84) because of its superior specificity and sensitivity.

First, we compared the staining properties of mAb 4H84 with the mAbs we produced previously (1B8, 3F6) (11). Frozen sections of second trimester placenta and placental bed samples were double stained with an anti-cytokeratin Ab (7D3), which reacts

Abbreviations used in this paper: PNGase F, peptide-N-glycosidase F.
with all trophoblast cell types (Fig. 1A), and 4H84 (Fig. 1B). As we reported earlier, extravillous invasive cytotrophoblasts in the uterine wall (placental bed) stained strongly for HLA-G throughout pregnancy. No immunoreactivity was detected in any cells of floating villi (syncytiotrophoblast, villus cytotrophoblast, villus core, endothelium, fetal blood cells). These results suggested that 4H84 had the same staining characteristics as the anti-HLA-G Abs we originally produced (11). In our previous study, we did not examine HLA-G expression in extraembryonic membranes. In this study, we double stained frozen sections of second trimester (18 wk) amnion-chorion with 7D3, to distinguish amniocytes and cytotrophoblasts (Fig. 1, C and E), and 4H84. All cells of the amnion layer reacted with the mAb that specifically recognized HLA-G (Fig. 1D). Many of the multilayered cytotrophoblasts within the chorion also reacted with the Ab, but not all cells stained with equal intensity, and some cells failed to demonstrate any immunoreactivity (Fig. 1F). In addition, a few cells that did not express cytokeratin reacted weakly with 4H84.

**Immunoblot characterization of the HLA-G heavy chain protein produced by placental villi, invasive cytotrophoblasts, and JEG choriocarcinoma cells**

SDS-PAGE and immunoblotting of detergent extracts of early gestation chorionic villi (12 wk) showed the HLA-G heavy chains as heterodisperse immunoreactive bands smeared across a m.w. range from approximately $M_r$ 39,000–48,000 (Fig. 2). Conditioned medium from first trimester (12 wk) placental villi cultured for 48 h contained bands that spanned a similar m.w. range and also included isoforms of slightly lower m.w. These results were typical of those we obtained from early gestation samples, although some contained isoforms of even higher m.w. Analysis of extracts prepared from term placental villi showed that they contained less HLA-G per milligram of protein, and that the bands detected were less heterodisperse. We then investigated whether a cytotrophoblast cell line (JEG-3) that produces HLA-G makes a similar array of heavy chain proteins. 4H84 reacted with an $M_r$ 40,000–43,000 protein in extracts prepared from JEG cells (Fig. 2). In contrast, the major band identified in conditioned medium from these cells was an $M_r$ 37,000–40,000 protein. Both of these bands displayed much less m.w. heterogeneity than those detected in placenta-derived samples. We also investigated the nature of the HLA-G heavy chains produced by primary cultures of purified first trimester cytotrophoblasts that were allowed to differentiate along the invasive pathway in vitro. Both cell extracts (Fig. 4) and CM samples (data not shown) contained heterodisperse immunoreactive bands that resembled those detected in samples of placental villi rather than the HLA-G produced by JEG cells.
acted only with the recombinant protein. As a positive control we included a recombinant Gsol sample, and as a negative control we analyzed PBL extracts. Among these samples, the mAb 16G1 reacted strongly with the recombinant protein, but failed to react with cell extracts or conditioned medium from placental samples and have never seen cross-reactivity with other classical class I (Ia) molecules on maternal and nontrophoblast fetal cells within these specimens. Thus, although it is not technically feasible to test this mAb for cross-reactivity against all alleles, 4H84 does not appear to cross-react with the commonly expressed classical class I molecules.

Placental HLA-G does not contain epitopes encoded by intron 4

Previous reports suggested that soluble HLA-G is the product of an alternatively spliced mRNA that contains intron 4. This mRNA has a stop codon 21 amino acids after the α3 domain, thus excluding the transmembrane region. LCL.221 cells transfected with a construct that expresses this mRNA released the corresponding protein, Gsol (17). To determine whether the Gsol molecule contributed to the m.w. heterogeneity of the placental HLA-G heavy chains, we analyzed placental cell extracts and conditioned medium by immunoblotting with mAb 16G1 (Fig. 3). This mAb was raised against a synthetic peptide corresponding to a portion of the intron 4 sequence. A recombinant Gsol sample served as a positive, and PBL extracts as a negative control. Among these samples, the mAb 16G1 reacted only with the recombinant protein.

It was also important to know whether 4H84 cross-reacted with classical MHC class I molecules expressed by other cells. A sample of a detergent lysate of PBL obtained from a single individual contained no 4H84-immunoreactive proteins (Fig. 2). PBLs from at least 20 other individuals were analyzed with identical results (data not shown). In addition, we have used this mAb for immunolocalization on tissue sections prepared from at least 30 different placental samples and have never seen cross-reactivity with other class I (la) molecules on maternal and nontrophoblast fetal cells within these specimens. Thus, although it is not technically feasible to test this mAb for cross-reactivity against all alleles, 4H84 does not appear to cross-react with the commonly expressed classical class I molecules.

The array of placental HLA-G isoforms is primarily due to glycosylation

The broad m.w. range of the HLA-G-immunoreactive bands in placental villi and cytotrophoblast samples could be due to the presence of other splice variants of the molecule or to heterogeneous glycosylation. To assess the role of glycosylation, we treated cytotrophoblast and JEG-3 cell lysates with PNGase F, an asparagine amidase that releases N-linked oligosaccharide chains, and then analyzed the products by immunoblotting with 4H84. The results are shown in Figure 4A. After N-deglycosylation, the Ab primarily reacted with a more discrete band (M, 37,000–42,000) in cytotrophoblast extracts; a great deal of the smearing previously observed was eliminated. This band had an electrophoretic mobility that was similar to HLA-G in JEG cell extracts that had also been treated with PNGase F. Likewise, enzymatic N-deglycosylation of HLA-G in extracts of early gestation chorionic villi resulted in a single major band of M, 37,000–39,000. PNGase F treatment of conditioned medium from villus explant cultures revealed a prominent band that comigrated with cell-associated HLA-G. A slightly lower m.w. band, migrating as a doublet in some samples, was consistently detected and presumably represents the soluble form of the molecule.

Placental cells do not translate into protein an alternatively spliced HLA-G mRNA that contains intron 4. Placental cell extracts and conditioned medium were analyzed by immunoblotting with mAb 16G1, raised against a synthetic peptide corresponding to a portion of the intron 4 sequence. A recombinant Gsol sample served as a positive, and PBL extracts as a negative control. Among these samples, the mAb 16G1 reacted only with the recombinant protein.

Placental HLA-G m.w. heterogeneity is due primarily to glycosylation. A, JEG and cytotrophoblast cell extracts were analyzed by immunoblotting without treatment (−) or after digestion (+) with either PNGase F, which N-deglycosylates the molecule, or endo-β-D-galactosidase (Endo-β-Gal), which removes polylactosamine chains, leaving the oligosaccharide core intact. In comparison with HLA-G in JEG cell extracts, removal of oligosaccharide units from placental HLA-G significantly reduced the m.w. heterogeneity of the molecule. B, Enzymatic N-deglycosylation of HLA-G in extracts of early gestation chorionic villi similarly resulted in a single major band of M, 37,000–39,000. PNGase F treatment of conditioned medium from villus explant cultures revealed a prominent band that comigrated with cell-associated HLA-G. A slightly lower m.w. band, migrating as a doublet in some samples, was consistently detected and presumably represents the soluble form of the molecule.
hypothesis, we treated cytotrophoblast samples with endo-
reminiscent of the polylactosaminylated placental glycoproteins and cytotrophoblast samples had a ladder-like appearance that was The 4H84-immunoreactive bands observed in the chorionic villus chains when they are isolated from other cellular sources (24, 25).

PNGase F collapsed the broad bands detected in the untreated samples into a prominent M, 35,000–36,000 band. Most samples also contained a much less intense band corresponding to the mobility of the cell-associated form of the molecule (~M, 38,000). The faint M, 47,000 band in the enzyme-treated samples was due to nonspecific reactivity with the secondary Ab.

FIGURE 5. Highly glycosylated HLA-G isoforms are detected in amniotic fluid by immunoblotting. Lanes 1 and 2 contained early gestation samples (5 μl); lanes 3 and 4 contained third trimester samples (5 μl). Treatment of the samples with PNGase F collapsed the broad bands detected in the untreated samples into a prominent M, 35,000–36,000 band. Most samples also contained a much less intense band corresponding to the mobility of the cell-associated form of the molecule (~M, 38,000). The faint M, 47,000 band in the enzyme-treated samples was due to nonspecific reactivity with the secondary Ab.

Our previous work shows that trophoblasts add polylactosamine carbohydrate chains (repeating units of either Galβ1,3GlcNAc (type 1) or Galβ1,4GlcNAc (type 2)) to fibronectin and certain integrin receptors, molecules that carry simple bi- and triantennary chains when they are isolated from other cellular sources (24, 25). The 4H84-immunoreactive bands observed in the chorionic villus and cytotrophoblast samples had a ladder-like appearance that was reminiscent of the polylactosaminylated placental glycoproteins we described previously (24). This suggested that HLA-G from placent al sources may carry this unusual modification. To test this hypothesis, we treated cytotrophoblast samples with endo-β-D-galactosidase (V-Labs), an enzyme that hydrolyzes polylactosamine oligosaccharide units, but leaves the rest of the saccharide core intact. This treatment increased the mobility of placent al HLA-G and decreased the molecular mass heterogeneity and ladder-like appearance of the bands (Fig. 4A). As expected from treating the samples with this enzyme, which only partially deglycosylates the molecule, the endo-β-D-galactosidase-treated HLA-G was intermediate in mobility between untreated and PNGase-treated samples. The mobility of JEG-3 HLA-G was unaffected by treatment with this enzyme, indicating that this cell line does not add polylactosamine units to the oligosaccharides it carries.

HLA-G isoforms are detected in amniotic fluid

We have been very interested in the possibility that biologic fluids of either maternal or fetal origin contain HLA-G. Given the pattern of HLA-G protein expression in the amnion-chorion, it seemed likely that this molecule might be detected in amniotic fluid. To test this hypothesis, 30 samples were collected from women undergoing amniocentesis for prenatal genetic testing at 16 to 18 wk of pregnancy; 10 samples were obtained from women undergoing amniocentesis for fetal maturity assessment in the third trimester of pregnancy. By immunoblot analysis, we detected HLA-G in every sample. Representative results are shown in Figure 5. 4H84 reacted with bands of varying intensity that had different degrees of m.w. heterogeneity in all of these samples. These differences did not correlate with gestational age. Treatment of the samples with PNGase F collapsed the broad bands detected in the untreated samples into a prominent M, 35,000–36,000 band corresponding to the estimated m.w. of the secreted form of HLA-G detected in placent al villus conditioned medium. A much less intense band, corresponding to the mobility of the cell-associated form of the molecule (~M, 38,000), was also detected in most of these samples. The faint M, 47,000 band in the enzyme-treated samples was due to nonspecific reactivity of the secondary Ab used in this experiment. As with the placental samples, endo-β-D-galactosidase treatment of amniotic fluid resulted in a significant reduction in m.w. heterogeneity of the immunoreactive bands (data not shown).

The presence of HLA-G in amniotic fluid suggested that the molecule might be detected in the cervical mucus after membrane rupture. To test this hypothesis, we collected cervical mucus from 10 women in labor. The cotton swabs used to collect the specimens were extracted with SDS-PAGE loading buffer; the dissolved samples were then electrophoretically separated and transferred to nitrocellulose. Representative examples of the immunoblot analyses are shown in Figure 6. We readily detected HLA-G in the cervicovaginal fluid of six of seven women with ruptured membranes. In all cases, the bands had less m.w. heterogeneity than was observed in the amniotic fluid samples. HLA-G was not detected in three samples from women with intact membranes.

Discussion

The discovery that HLA-G is expressed by the human placenta represents a major advance in our understanding of factors that govern interactions between maternal and fetal cells during pregnancy. In this regard, knowing the exact location of the placental cells that express this Ib molecule is important for formulating hypotheses about how HLA-G functions. As to which of the component cells of the organ express HLA-G, most published studies suggest that only its specialized epithelial cells, termed cytotrophoblasts, synthesize HLA-G mRNA and protein (11, 26).

Another layer of complexity is added to the interpretation of the immunolocalization data by the fact that whereas cytotrophoblasts are found in three different locations, in only two of these sites do they express HLA-G. With regard to the first location, our previous immunolocalization study with an anti-HLA-G IgM Ab showed that cytotrophoblast stem cells anchored to the basement membranes of chorionic villi do not express this class Ib molecule. But extravillous cytotrophoblasts that detach from this basement membrane and migrate through the columns that connect the placenta to the uterus up-regulate HLA-G expression. Expression in this second location is maintained once the cells have reached their final destination: either the interstitium of the uterine wall or the maternal vessels that traverse this region (11). Our findings have since been confirmed by a different group using yet another anti-HLA-G Ab (26). The fact that the new IgG Ab described in this work (4H84) failed to react with cytotrophoblast stem cells, but stained the extravillous population, suggests that it has the same specificity as the previously described Abs.
Much less is known about cytotrophoblast expression of HLA-G in the third location, the amnion-chorion. The possibility that this subpopulation of cells expresses HLA-G was first suggested by the work of Ellis et al. (7), who found, by using Northern hybridization, that cytotrophoblasts isolated from the amnion-chorion expressed HLA-G mRNA. Subsequently, other studies confirmed this finding (9) and showed that amniotic epithelia also express this mRNA (27). To our knowledge, the present study is the first report of the results of immunolocalization experiments using an HLA-G-specific Ab to study expression of this Ag in the amnion-chorion. In accordance with the mRNA data, we found that both cytotrophoblasts and amniocytes stained brightly with mAb 4H84. This prompted us to consider whether HLA-G, produced by either cell type, may also be a component of amniotic fluid. Immunoblot analyses, performed with the same Ab, confirmed this hypothesis. Taken together, our studies of HLA-G expression in various locations within the placenta and the amnion-chorion suggest that cytotrophoblasts that are in direct contact with maternal tissues express this class Ib molecule. In addition, we now know that the fetus is also surrounded by cells (i.e., amniocytes) that express, and fluid that contains, HLA-G.

We are interested in using the 4H84 mAb and the results of the studies reported in this work to design experiments to understand how HLA-G affects the maternal, and possibly the fetal, immune response during pregnancy. In this regard, our immunoblotting data suggest that the biologic activity of this molecule resides in either the full-length protein or a previously described truncated form (8), the exact origin of which remains elusive. We found no evidence that placental cells produce a soluble protein encoded by an HLA-G mRNA species that contains intron 4. Likewise, we failed to detect any immunoreactive bands corresponding to the predicted m.w. of the proteins that would be encoded by the other alternatively spliced mRNAs that have been described (G2, M, 26,000; G3, M, 15,000; G4, M, 25,000; intron 4 HLA-G, M, 31,000). In support of this conclusion, extensive Northern hybridization experiments conducted in our laboratory and by other investigators failed to detect transcripts that correspond to the predicted size of these splice variants (8, 10, 11, 28, 29). Thus, it seems likely that the alternatively spliced mRNAs that are frequently detected using reverse-transcriptase PCR (reviewed in Ref. 12) are not translated into protein.

A number of recent studies have focused on the role that HLA-G plays in regulating the maternal immune response to the fetus. In vivo, cytotrophoblasts in two locations could be involved in this phenomenon. Whereas little is known about maternal leukocyte interactions with fetal cells in the amnion-chorion, a great deal of evidence suggests that the HLA-G-positive cytotrophoblasts within the uterine wall are in direct contact with the unusual maternal NK cells that reside in this location. Cell-cell interactions within the uterine wall have been simulated in vitro by coculturing LCL/221 cells that overexpress HLA-G with peripheral or decidual NK clones. Using this approach, several groups have identified HLA-G-specific inhibitory receptors on NK cells (30–33). For example, Soderstrom and colleagues recently showed that HLA-G expressed on target cells inhibits decidual NK cell killing by interacting with the CD94/NKG2 inhibitory receptor. This inhibitory receptor is a lectin-type molecule, raising the possibility that the types of carbohydrate chains HLA-G carries might be relevant to its biologic activity. Thus, it is interesting to note that HLA-G carries an unusual type of glycosylation. Like all other human class I molecules, it contains a single N-linked glycosylation site (Asn69). But here the similarities end. Whereas the MHC class Ia molecules carry simple biantennary structures (34), HLA-G oligosaccharides are modified by the addition of numerous polylactosamine units. We hypothesize that such a modification could increase the stability of the molecule in the proteinase-rich environment of the uterus. This appears to be one function of the polylactosaminylated saccharides carried by placental fibronectins (24). Additionally, we are intrigued by the possibility that the unusual glycosylation HLA-G carries might enhance its ability to interact with the CD94/NKG2 inhibitory receptor.

The finding that HLA-G, produced by chorionic cytotrophoblasts and amniocytes, is easily detected in amniotic fluid raises the additional possibility that this class Ib molecule could function during development of the fetal immune system. In this regard, it is interesting to note that, beginning in early pregnancy, the fetus swallows amniotic fluid. Oral exposure is known to have efficacy in reestablishing tolerance to Ags that are implicated in autoimmune diseases, such as multiple sclerosis (35) and diabetes (36). Whether this route of exposure could explain how tolerance to HLA-G is established remains to be determined.

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