Human leukocyte antigen-G in the male reproductive system and in seminal plasma

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Abstract: One of the non-classical human leukocyte antigen (HLA) class Ib proteins, HLA-G, is believed to exert important immunoregulatory functions, especially during pregnancy. The presence of HLA protein in paternal seminal fluid has been suggested to have an influence on the risk of developing pre-eclampsia. We have investigated whether HLA-G protein is present in human seminal plasma and in different tissue samples of the male reproductive system. Western blot technique and a soluble HLA-G (sHLA-G) assay were used to detect sHLA-G in human seminal plasma samples. Immunohistochemical staining was performed on paraffin-embedded tissue samples. We detected sHLA-G protein in seminal plasma, and HLA-G expression in normal testis and in epididymal tissue of the male reproductive system but not in the seminal vesicle. Furthermore, the results indicated a weak expression of HLA–G in hyperplastic prostatic tissue. In summary, several of the findings reported in this study suggest an immunoregulatory role of HLA-G in the male reproductive system and in seminal plasma.

Key words: MHC / HLA-G / reproduction / seminal plasma / male reproductive system

Introduction

The non-classical human leukocyte antigen (HLA) class Ib molecule, HLA-G, has a restricted pattern of expression but is expressed at the feto–maternal interface, especially on extravillous cytotrophoblasts and amnion epithelial cells. HLA-G seems to be an integral part of placental development as an inducer of immunological tolerance, allowing the implantation of the semi-allogenic embryo into the maternal tissues. HLA-G is expressed both as membrane bound (HLA-G1, -G2, -G3 and -G4) and soluble isoforms (HLA-G5, -G6 and -G7). Furthermore, HLA-G molecules can be shed or proteolytically cleaved from the cell surface, so-called soluble HLA-G1 (sHLA-G1) and sHLA-G2, which correspond to the soluble HLA-G5 and HLA-G6 isoforms, respectively. Soluble HLA-G5 and -G6 are generated by the retaining of intron 4, which includes a stop codon, in the HLA-G transcript (Kovats et al., 1990; Hviid et al., 1998; Hunt et al., 2005; Hviid, 2006; Carosella et al., 2008).

Only a few studies have focused on HLA-G expression in the male reproductive system (Ryan et al., 2002; Langat et al., 2006). For at least two reasons, it can be hypothesized that HLA-G might be present in male reproductive tissues. First, HLA-G has been detected in immune-privileged sites such as the placenta and the eye (e.g. Le Discorde et al., 2003). In this regard, the testis is also considered an immune-privileged site. Secondly, seminal plasma seems to have an immunological ‘priming’ effect in the female before implantation of the embryo (Robertson, 2005).

Pre-eclampsia is a multisystem disorder that usually develops in the second or third trimester of pregnancy, and can have fatal consequences. The involvement of some kind of immune maladaptation is one of the main hypotheses of the etiology of pre-eclampsia. Polymorphisms in the HLA-G gene have been associated with the risk of developing pre-eclampsia (Hylenius et al., 2004; Moreau et al., 2008). Furthermore, previous studies have shown that sperm, and probably HLA in the seminal fluid, in some way might have an influence on the incidence of pre-eclampsia (Koelman et al., 2000; Robertson et al., 2003; Saito et al., 2007). Therefore, it can be hypothesized that paternal sHLA molecules, perhaps including HLA–G, in the seminal plasma, may be involved in inducing tolerance in the
mother to paternal antigens. This induction of allogeneic tolerance to paternal HLA molecules of the fetus may be important for the success of the pregnancy.

A few studies on HLA class Ib gene expression in male gametogenic cells (Fiszer et al., 1997) and on immature and mature human germ cells (Hutter and Dohr, 1998) have been reported. However, the only studies performed so far within male reproductive systems, in which the focus of the investigation being HLA-G, or a HLA-G homolog gene, has been in the rhesus monkey (Ryan et al., 2002), and in human male prostate (Langat et al., 2006). Ryan et al. (2002) investigated the expression of the non-classical major histocompatibility complex class I molecule Mamu-AG (a homolog gene to human HLA-G) in different anatomical sites in male rhesus monkeys. Ryan et al. (2002) detected Mamu-AG-S mRNA coding for soluble Mamu-AG in rhesus testis (Ryan et al., 2002). A monoclonal antibody (mAb) against membrane-bound Mamu-AG stained a few cells in the testis. Also, it was found that Sertoli cells, spermatocytes and spermatids were consistently positive for immunostaining with 16G1, which is a mAb against the retained intron 4 in soluble HLA-G5. Finally, the presence of Mamu-AG-S in the syncytiotrophoblasts of the chorionic villi of the rhesus placenta was observed. By RT–PCR, Langat et al. (2006) found mRNAs specific for HLA-G1, -G2, -G5 and -G6 in samples of normal prostate gland and prostate adenocarcinomas. Furthermore, by using immunohistochemical staining they observed that out of the four HLA-G mRNA isotypes, only HLA-G5 protein was present in the prostate and prostatic secretions. Prostatic adenocarcinoma tumor cells showed almost an absence of the HLA-G5 protein, although the protein remained detectable in the secretions (Langat et al., 2006).

The aim of this present study was to investigate whether there are any sHLA-G in seminal plasma (defined as the liquid supernatant after centrifugation of semen) and/or in semen (seminal fluid containing spermatozoa). Furthermore, the aim was also to investigate whether HLA-G is present in tissue samples from testis, prostate, epididymis, ductus deferens and seminal vesicles of the male reproductive system. The presence of HLA-G in the male reproductive system and in semen could play a role in induction of immunological tolerance in the fertile female to paternal antigens and thereby for the success of pregnancy.

### Materials and Methods

**Antibodies**

The monoclonal HLA-G antibodies MEM-G/1, MEM-G/2, 2A12 and 5A6G7 were used to detect HLA-G. All mAbs were obtained from Exbio Praha (Prague, Czech Republic). MEM-G/1 and MEM-G/2 are mouse monoclonal [immunoglobulin G1 (IgG1)] antibodies that recognize the free heavy chains of all the HLA-G isoforms by western blot and paraffin-embedded immunohistochemistry. The 2A12 and 5A6G7 mAbs (mouse IgG1) are antibodies raised against the intron 4-retaining protein sequence of sHLA-G. Thus, these two antibodies are developed to discriminate between sHLA-G protein yielded by shedding from membrane-bound HLA-G isoforms that do not contain the intron 4-encoded protein sequence and the recognition of soluble HLA-G5/HLA-G6 produced from the intron 4-retaining alternatively spliced mRNAs. All the mAbs used in the study are listed in Table I.

**Samples and tissues**

The following different tissue samples were included in the immunohistochemical analyses: endometrium from abortion tissue (6th week of gestation; one sample), normal placenta (39th week of gestation; one sample), normal testis (three samples), testis with atrophy (one sample), prostate with hyperplasia (seven samples), normal epididymis (five samples), normal ductus deferens (four samples) and normal seminal vesicle (four samples).

**Preparation of seminal samples for immunohistochemistry**

Semenal samples and separated sperm cells from four donors were immobilized in a coagulation gel and fixed in formalin before being paraffin-embedded for immunohistochemistry. The seminal samples were used directly or the sperm cells and other cells were separated by centrifugation. Centrifugation was performed at 1000g for 10 min at 4°C.

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**Table I Monoclonal antibodies used in this study for the detection of different HLA-G isoforms in different applications.**

<table>
<thead>
<tr>
<th>Antibody clone</th>
<th>Specificity</th>
<th>Application</th>
<th>Ig-Isotype</th>
<th>Dilution (IHC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM-G/9</td>
<td>HLA-G1 and HLA-G5 (conformational)</td>
<td>ELISA</td>
<td>IgG1</td>
<td>—</td>
</tr>
<tr>
<td>MEM-G/1</td>
<td>All HLA-G isoforms (denatured)</td>
<td>IHC(P), WB</td>
<td>IgG1</td>
<td>1:750</td>
</tr>
<tr>
<td>MEM-G/2</td>
<td>All HLA-G isoforms (denatured)</td>
<td>IHC(P)</td>
<td>IgG1</td>
<td>1:100 (and 1:2000)</td>
</tr>
<tr>
<td>5A6G7</td>
<td>HLA-G5 and HLA-G6 (anti peptide, intron-4 retaining alternatively spliced mRNAs)</td>
<td>IHC(P)</td>
<td>IgG1</td>
<td>1:50 (and 1:500)</td>
</tr>
<tr>
<td>2A12</td>
<td>HLA-G5 and HLA-G6 (anti peptide, intron-4 retaining alternatively spliced mRNAs)</td>
<td>IHC(P)</td>
<td>IgG1</td>
<td>1:500</td>
</tr>
<tr>
<td>PPV-06</td>
<td>A plant pathogen</td>
<td>IHC(P)</td>
<td>IgG1</td>
<td>1:50</td>
</tr>
<tr>
<td>MOPC-21</td>
<td>Unknown specificity</td>
<td>IHC(P)</td>
<td>IgG1</td>
<td>1:50</td>
</tr>
<tr>
<td>B2M-01</td>
<td>Beta 2-microglobulin associated with HLA class I antigens</td>
<td>ELISA</td>
<td>IgG2a</td>
<td>—</td>
</tr>
<tr>
<td>X16/99</td>
<td>CD45, pan-leucocyte marker</td>
<td>IHC(P)</td>
<td>IgG1</td>
<td>1:50</td>
</tr>
</tbody>
</table>

IHC(P), immunohistochemistry paraffin sections; ELISA, enzyme-linked immunosorbent assay; WB, western blot.

*Specificity according to the manufacture and Menier et al. (2003). It is demonstrated in the publication by Menier et al. (2003) that MEM-G/1 and MEM-G/2 can detect all denatured isoforms of HLA-G in western blotting. However, in the case of immunohistochemistry, it is only shown that MEM-G/1 stains extravillous trophoblast cells in paraffin-embedded sections of human first-trimester placenta tissue. According to information obtained from the manufacture (Exbio Praha), both MEM-G/1 and MEM-G/2 stain paraffin-embedded trophoblast cells but other tissues with weak HLA-G expression are stained only with MEM-G/2.

*All antibodies are acites formulars.*
followed by the removal of the seminal plasma. The seminal plasma was stored at −20°C until ELISA was performed. The cells from the seminal sample were used directly or washed two times by resuspending them in 2 ml of 0.9% NaCl followed by centrifugation steps at 800g for 10 min at 4°C. Leukocytes from a normal human donor were used as a control. A sample of 100 μl blood diluted in 0.9% NaCl was lysed using 200 μl lysis buffer diluted in 1800 μl water. After 10 min, the leukocytes were washed two times by resuspending them in 1.5 ml 0.9% NaCl followed by centrifugation at 800g for 5 min at room temperature. Then, the cells were immobilized in a coagulation gel in the same way as for the raw seminal samples. Three drops of human plasma were added along with two drops of bovine thrombin. After 1 min, a coagulation gel complex had formed and this was fixed over night in 1 ml formalin. For the raw seminal samples a double volume of plasma and thrombin was added.

**Immunohistochemistry**

Immunohistochemical studies were performed on paraﬁn-embedded sections using mAbs specific for HLA-G (MEM-G/1, MEM-G/2, 2A12, 5A6G7; Exbio Praha, Czech Republic) and negative control antibodies PPV-06 (mouse Ig-isotype matched mAb against a plant pathogen; Exbio Praha), and MOPC-21 (mouse Ig-isotype matched mAb; Exbio Praha). Briefly, the sections were dewaxed in xylene before immunostaining and placed in 99% ethanol. Endogenous peroxidase activity was quenched with 0.5% H2O2 in 99% ethanol for 20 min, after which sections were washed in tap water. For pretreatment with heat-induced epitope retrieval, the slides were placed in a 10.0 mM Tris and 0.5 mM EGTA buffer (pH 8.7–8.8) and microwaved in a domestic microwave oven at the maximum power (900 W) for 10 min followed by heating for 20 min at the medium power (400 W). Slides were subsequently kept in buffer for 10 min to cool. After pretreatment, slides were washed in tap water, followed by washes in 0.05 M Tris–HCl wash buffer (pH 7.6) containing 0.05% Tween 20 (Dako, Glostrup, Denmark). The slides were incubated with primary antibodies (see Table I) diluted in Power Block solution (BioGenex, San Ramon, CA, cat.no. HK085-5K) for 30 min at room temperature. The sections were then rinsed in the wash buffer (3 × 5 min), and the reactions were detected with Super Sensitive polymer-horseradish peroxidase (HRP) reagent (BioGenex, cat.no. QD410D-XC) and viewed with LumiGLO Reserve Chemiluminescent Substrate kit (KPL, Gaithersburg, MD, USA) was carried out. Bands were viewed by the use of a LAS-4000 Luminescent Image Analyzer (Fujifilm Life Science, Stamford, CT, USA) for varying exposure times ranging from 10 s to 5 min to ascertain the most optimal pictures.

**Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blotting**

Seminal plasma samples for western blots were diluted 1:2 by mixing 10 μl of seminal plasma with 10 μl sample buffer (sample buffer: Laemmli 2× concentrate containing 4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris–HCL, pH 6.8 (Sigma-Aldrich, Steinheim, Germany)). Afterwards samples were boiled for 5 min at 99°C. Each gel was loaded with a standard protein of broad Prestain Molecular Weight Marker, 7.6–195 kDa (Two-color SDS Marker, PAGegel, San Diego, USA). Afterwards, samples were loaded on a precast 12% gradient 12-well SDS polyacrylamide gel (PAGEgel). Proteins were then separated by electrophoresis and were transferred to nitrocellulose membranes (Pierce, Rockford, USA) using a Mini Trans-Blot Cell apparatus (Bio-Rad, Life Science Research, Denmark), according to the manufacturer’s instructions for blotting. The membranes were blocked at room temperature for 45 min in blocking buffer containing 5% skim milk powder (Merck, Darmstadt, Germany) dissolved in 1× PBS. The membranes were washed with wash buffer containing 1× PBS with 5% (bovine serum albumin/fraction V) and incubated over night at room temperature, with primary antibody MEM-G1 in a concentration of 10 μg/ml (recommended dilution by the antibody supplier, Exbio, Praha), diluted in wash buffer. The membranes were washed with the wash solution. Then, membranes were incubated for 2 h at room temperature with secondary polyclonal rabbit anti-mouse immunoglobulins conjugated with HRP (Dako) diluted 1:1000 in wash buffer. After the incubation with conjugate, membranes were again washed four times for 10 min in wash solution. After incubation, detection using LumiGLO Reserve Chemiluminescent Substrate kit (KPL, Gaithersburg, MD, USA) was carried out. Bands were viewed by the use of a LAS-4000 Luminescent Image Analyzer (Fujiﬁlm Life Science, Stamford, CT, USA) for varying exposure times ranging from 10 s to 5 min to ascertain the most optimal pictures.

**Soluble HLA-G ELISA**

To quantify sHLA-G in seminal plasma, we used a commercially available ELISA kit (Exbio/BioVendor, Praha) that uses the mAb MEM-G/9 specific for sHLA-G1 and HLA-G5. Six diluted human seminal plasma samples from male donors were loaded manually into the wells and incubated the study was carried out in accordance with the ethical standards of the Helsinki Declaration and was approved by the Committee on Biomedical Research Ethics of the Zealand Region of Denmark.

**Preparation of seminal plasma and normal sperm samples for western blotting**

Normal semen was collected from male donors by masturbation. The semen was allowed to liquefy at room temperature for an hour and afterwards divided into 9 ml microfuge tubes (Nunc, Roskilde, Denmark). Seminal plasma was separated from the sperm by centrifuging at 1000g for 10 min at 4°C and stored at −20°C until electrophoresis was performed. Each aliquot of sperm (precipitate) was then washed by suspension in 2 ml phosphate-buffered saline (PBS; pH 7.4) and then centrifuged at 800g for 10 min at 4°C. The supernatant was discarded. The sperm aliquots were subsequently suspended in 300 μl of protease inhibitor cocktail buffer (Sigma, Missouri, USA) and stored at −80°C until use. As a negative control, semen from a boar (Hatting-KS, Denmark) was used; this was prepared in the same way as the human semen samples. Furthermore, human kidney tissue lysate (Abcam, Cambridge, UK) was used, also as a negative control. Human placenta whole cell lysate (Abcam, Cambridge, UK) was included as a positive control.
for 20 h. The remaining procedure was programmed into and carried out using the automated analyzer BEP2000 (Dade Behring) according to the manufacturer’s instructions. As negative controls, both a blank and a sample with boar seminal plasma were used. All samples were tested in duplicates. Soluble HLA-G standard was diluted to obtain a calibrator curve within a range from 3.91 to 125 U/ml for sHLA-G ELISA. Results were adjusted for the sample dilution. The total protein concentration levels were quantified in the seminal plasma samples by using an ARCHI-TECT c8200 Integrated System (Abbott Diagnostics). Afterwards, results from the ELISA were adjusted by dividing sHLA-G concentration (U/ml) with total protein concentration (mg/ml).

**Results**

**The presence of HLA-G in different parts of the human male reproductive system**

Immunohistochemical analysis of HLA-G was performed in the tissues listed in Materials and Methods; all paraffin-embedded tissue slides. Representative immunohistochemical sections are presented in Figs 1–5. As negative controls, the staining procedure was performed with two different Ig-isotype-matched nonsense antibodies. No positive staining pattern was seen on the negative controls [example shown in Fig. 1 (insert)]. HLA-G1 is expressed on the surface of extravillous trophoblast cells and HLA-G5 produced in extravillous trophoblast cells (e.g. Hunt and Langat, 2009), and therefore placental or endometrial (abortion) tissue slides were used as positive controls for the mAbs MEM-G/1, MEM-G/2, 5A6G7 and 2A12.

Results of the staining of first trimester placental (abortion) tissue from gestational week 6 by the four mAbs are shown in Fig. 1. As expected, MEM-G/1, MEM-G/2 and 5A6G7 showed positive staining of extravillous trophoblast. MEM-G/1 and MEM-G/2 did not stain endometrial epithelial cells, as was the case for 5A6G7. The same staining patterns (of extravillous trophoblast) of these three mAbs have been reported before in the literature (Menier et al., 2003; LeMaoult et al., 2005; Helige et al., 2008). For the MEM-G/1 and MEM-G/2 mAbs, no change in staining intensities and patterns were observed, when the 22-mer HLA-G intron 4 peptide was added in absorption control experiments, and this was expected (Fig. 5). Furthermore, staining patterns of endometrial tissue are shown in Fig. 1 with the two intron-4-specific antibodies (2A12 and 5A6G7). Both the 2A12 and 5A6G7 mAbs stain endometrial epithelial cells, but only 5A6G7 stains extravillous trophoblast. The peptide absorbing experiments showed that the staining with the 5A6G7 mAb is absolutely blocked in all tissues tested (abortion tissue, testis, epididymis and prostate). In the testis and in the prostate tissues the staining by 2A12 is totally blocked; however, in the abortion tissue and in the epididymis sections, where the staining without peptide is very intense, some weak staining remains (Fig. 5).

Positive reactions with the 2A12 and 5A6G7 mAbs, respectively, were observed in the testis (see Fig. 2). The results from the testicular tissue indicate that some of the Sertoli cells in the testis stain positive when HLA-G antibodies raised against the sHLA-G isoforms are tested. However, the testicular tissue showed no positive staining with the use of the two other HLA-G antibodies, MEM-G/1 and MEM-G/2.

![Figure 1](https://example.com/f1.png)  
**Figure 1** Immunohistochemistry, using different antibody clones against HLA-G, clone 2A12 and 5A6G7 [soluble isoforms of HLA-G (HLA-G5 and -G6)], clone MEM G/1 and MEM G/2 [all denatured isoforms of HLA-G], in human paraffin-embedded endometrial (abortion) tissue from week 6 of gestation. **(A)** 2A12 shows positive staining of endometrial epithelial cells (EECs), while the extravillous trophoblasts (EVT) are negative. **(B)** 5A6G7 shows positive staining of EEC as well as of EVT. Insert: negative control. **(C and D)** MEM G/1 and MEM G/2 shows only positive staining of EVT, while staining of EEC are negative (Magnification ×200).
Figure 2 Immunohistochemical staining of HLA-G, clone 2A12 and 5A6G7 (against soluble isoforms of HLA-G), in human paraffin-embedded normal testis tissue. (A and B) Both 2A12 and 5A6G7 show focal positive staining of Sertoli cells (Magnification ×200) (C) As (B) (Magnification ×600).

Figure 3 Immunohistochemical staining of HLA-G, clone 2A12, 5A6G7, MEM G/1 and MEM G/2, in human paraffin-embedded epididymis tissue. (A and B) 2A12 and 5A6G7 against soluble isoforms of HLA-G show positive staining of the cells of the ductuli efferentes. (C and D) No staining was observed with MEM G/1 and MEM G/2 (against all denatured isoforms of HLA-G) (Magnification ×200).
The same was observed with the paraffin-embedded epididymal tissue (Fig. 3). Again, no positive reaction with MEM-G/1 and MEM-G/2 was observed, whereas 2A12 and 5A6G7 reacted with some of the cells in epididymis, especially the ductuli efferentis (the small seminal ducts leading from the testis to the head of the epididymis).

In tissue samples from the prostate gland, there was a positive reaction with 5A6G7 but no positive reactions with the three other antibodies (Fig. 4). Finally, the seminal vesicle did not reveal any positive reactions with the four HLA-G-specific antibodies tested (results not shown).

In the four seminal samples that were embedded in a coagulation gel matrix and thereafter paraffin-embedded the staining of the pan-leukocyte marker CD45 clearly showed the presence of leukocytes. However, no staining was observed with the anti-HLA-G mAbs, MEM-G/1 and MEM-G/2, in any of the samples. For the 5A6G7 mAb against HLA-G5/-G6, the background versus specific signal had to be adjusted for optimal staining. With a dilution of 1:500, all cells in the seminal samples were negative. However, using this dilution, first trimester placental control tissue and epididymis were clearly positive, and testis was only very weak positive, indicating that sHLA-G expression in testis is less pronounced that in epididymis. The isotype controls were all negative. Representative examples of immunohistochemical staining of the seminal samples are shown in Fig. 6.

**Western blot analyses show the presence of sHLA-G in human seminal plasma**

The presence of sHLA-G in seminal plasma was investigated by western blotting, using the antibody MEM-G/1. In order to confirm the results, the western blot analysis was performed on at least three separate occasions and semen samples were at least from four different male donors. The western blot results for MEM-G/1 showed the same binding patterns within the different blots, confirming that results were reproducible. Placental lysate was used as a positive control with a HLA-G protein band of ≈39 kDa (corresponding to the HLA-G1 isoform), in agreement with the literature.

Representative results obtained in the western blot experiments are shown in Fig. 6. As seen in Fig. 7 (lanes 2–5), HLA-G protein bands were observed in both the human seminal plasma samples and in human placenta lysate. Also, a weaker HLA-G protein band was observed in samples of human semen (not shown). Western blot with isolated sperm cells was also performed but all were negative (results not shown). In Fig. 7, the human peripheral blood mononuclear cell (PBMC) lysate is positive (lane 7) demonstrating the presence of HLA-G in PBMCs. The negative controls, the boar seminal plasma and kidney lysate were negative in all blots made.

Protein bands with a molecular weight of ≈37 kDa with reactivity against MEM-G/1 were observed in all human seminal plasma samples. Thereby, the isotype of HLA-G present in seminal plasma is different from the isotype of 39 kDa observed in placenta (HLA-G1) (Kovats et al., 1990). According to the molecular weight, the protein bands in seminal plasma (and one of the PBMC lysates), correspond to the soluble HLA-G5 isoform.

**Soluble HLA-G is present in human seminal plasma in various concentrations**

Levels of sHLA-G (sHLA-G1/HLA-G5) were measured in six different human seminal plasma samples with the use of a sHLA-G ELISA.
Figure 5 Immunohistochemical control experiments. Absorbing the mAb with a 22-mer peptide corresponding to the C-terminal amino acid sequence of s HLA-G5 and -G6. (A–D) Endometrial (abortion) tissue (week 6 of gestation). Almost all staining by 2A12 is blocked. All staining by 5A6G7 is blocked. No interference with either the positive staining by the MEM-G/1 or the MEM-G/2 is observed. (E and F) All staining in testis by 2A12 and 5A6G7 is blocked. (G and H) Most staining by 2A12 in epididymis sections is blocked, and staining by 5A6G7 is blocked. (I and J) Positive staining by 5A6G7 in prostate sections is blocked by the 22-mer peptide.
Soluble HLA-G was detected in all seminal plasma samples (range 127 U/ml–2000 U/ml; and 4.0 U/mg total protein to 45 U/mg total protein) (Fig. 8). None of the negative controls showed any presence of sHLA-G. The results reveal that there is a high variability in seminal sHLA-G concentrations between male donors, also when these concentrations are standardized in relation to measurements of total protein in the samples.

For the four seminal samples analyzed in paraffin-embedded coagulation gels, the sHLA-G concentrations in the seminal plasma were in the range of 47–1580 U/ml (not shown in Fig. 8).

**Discussion**

This study provides new evidence for the presence of HLA-G protein in seminal plasma and in tissue samples from the testis, epididymis and a weak occurrence of HLA-G in the prostate gland of the male reproductive system.

Western blot was performed on different seminal plasma samples using MEM-G/1 as the primary anti-HLA-G mAb. In the western blot experiments (Fig. 7), placental lysate was used as a positive control and showed a HLA-G protein band corresponding to the full length HLA-G1 (39 kDa) protein isoform. In the seminal plasma, protein bands were ≏35–37 kDa corresponding to the soluble HLA-G5 isoform. The soluble HLA-G6 isoform has a molecular weight of 28 kDa (Le Rond et al., 2004) and bands corresponding to this protein size were not detected. These results were reproducible in all four seminal plasma samples. As negative controls both boar seminal plasma and human kidney lysate were used. The boar seminal plasma was chosen as a control to obtain comparisons of two biologically alike sample materials (seminal plasma). No expression of HLA-G is normally found in kidneys, for which reason also the kidney lysate was used as a negative control. Both the boar seminal plasma and the kidney lysate were negative in all western blots indicating that no cross-reactions or other unspecific binding by MEM-G/1 took place. Samples of whole semen showed a weak HLA-G band. Isolated

![Figure 6](image_url)  
Figure 6 Immunohistochemical staining of cells in seminal samples. Cells were immobilized in a plasma-thrombin gel and paraffin-embedded. (A) 5A6G7 (dilution 1:500; magnification ×200). (B) As (A) (Magnification × 600). (C) MEM-G/1 (dilution 1:750; Magnification × 200) (D) As (C) (Magnification × 600). (E) Isotype control PPV-06 (dilution 1:500; Magnification × 200) (F) Anti-CD45 staining of leukocytes (dilution 1:50; Magnification × 600).
sperm cells did not show any reactivity with the specific anti-HLA-G mAb. Thus, the weak protein band observed in samples of semen at 35–37 kDa is probably due to HLA-G protein found in the seminal plasma (results not shown). The observation of an HLA-G protein band in all seminal plasma samples is a very interesting and original finding. The intensity of the HLA-G protein bands was different between the male donors. The variations in intensities of HLA-G bands can be due to different concentrations of sHLA-G in each seminal plasma sample, and this indicates that levels of sHLA-G protein in seminal plasma are varying between male donors. These observations are in accordance with the sHLA-G ELISA results that showed different concentrations of sHLA-G in seminal plasma from different male donors. When corrected for total protein content a substantial variation in sHLA-G content was still observed (Fig. 8). In general, different levels of sHLA-G in blood samples have been detected from individual to individual in other studies. Such individual differences may at least partly be under genetic control (Hviid et al., 2003; Hviid et al., 2004; Chen et al., 2008).

Human seminal plasma is produced by contributions from the seminal vesicle, prostate (prostate secretions) and bulbourethral glands. The origin of the HLA-G5 protein found in the seminal plasma in this study is not yet known. However, it is likely that HLA-G5 found in the seminal plasma originates from prostate gland secretions. This would be in agreement with previous observations made by Langat et al. (2006) that HLA-G5 protein is expressed and located in the prostate and prostate secretions. It cannot be excluded whether HLA-G5 can be expressed in other parts of the male reproductive system from the study by Langat and co-workers since only HLA-G expression in the prostate gland was addressed. To obtain more information about this, we examined a possible presence of HLA-G in different parts of the male reproductive system by immunohistochemistry.

Staining with HLA-G-specific antibodies was carried out in different tissues from the male reproductive system (Figs 1–5). Endometrial abortion tissue was used as a positive control. As expected, the MEM-G/1 and MEM-G/2 mAbs showed a strong reaction pattern with HLA-G protein in extravillous trophoblast cells (Fig. 1). Furthermore, the 5A6G7 mAb, which is specific for the HLA-G5 and -G6 isoforms, clearly stained the extravillous trophoblast cells and also the endometrial epithelial cells (Fig. 1); the staining of the extravillous trophoblast cells in the seminal plasma samples. The levels of sHLA-G in the seminal plasma samples were determined by ELISA and corrected for total protein content. The results show a substantial variation in sHLA-G content between the male donors. The levels of sHLA-G vary from 127 to >2000 U/ml. Furthermore, standardized levels of sHLA-G in seminal plasma samples are shown as Units per mg total protein. Each sHLA-G result of the six individual samples is standardized and corrected by the total protein concentration. Values of sHLA-G in the six human seminal plasma samples are varying from 4.0 to >45.0 U/mg.
trophoblast is in accordance with that mentioned in the literature (LeMaoult et al., 2005). However, LeMaoult et al. apparently did not observe a positive reaction in the endometrial epithelial cells with the 5A6G7 mAb. The 2A12 mAb that has also been raised against the HLA-G5 and -G6 isoforms positively stained the endometrial epithelial cells but not the trophoblast cells in the current study (Fig. 1). The last finding was not expected and there is no obvious explanation for the differences in positive staining between the 5A6G7 and 2A12 mAbs. It would have been expected that the staining patterns by the 2A12 and 5A6G7 mAbs would have been the same. The discrepancy was observed in the abortion tissue, where 2A12 did not stain extravillous trophoblast. The two mAbs have been generated in the same way with the use of a 22-mer peptide corresponding to the C-terminal amino acid sequence of HLA-G5 and -G6 as immunogen. It has not been possible to find any reported use of the 2A12 mAb in the literature. Maybe the specificity could be slightly different between the two mAbs regarding the HLA-G5 and the HLA-G6 isoforms but this is pure speculation. When absorbing experiments with the 22-mer peptide corresponding to the C-terminal part of the HLA-G5 and -G6 was performed, the staining with the 2A12 mAb was not entirely blocked abortion tissue and epithelium, which stains very intensively with the 2A12 mAb, as it was with the 5A6G7 mAb. This could indicate a lesser degree of specificity for the 2A12 mAb but could also be a question of differences in sensitivity and concentrations in the absorbing experiments. We have not been able to find any descriptions of the staining patterns of the 2A12 mAb at the feto—maternal contact zone, or of other tissues, in the literature. In conclusion, the results obtained with the 2A12 mAb have to be evaluated very carefully, and in the current study it is chosen only to rely on the data obtained with the 5A6G7 mAb. The glandular epithelium of peritoneal endometriosis has been reported in one study to express HLA-G staining with the 4H84 mAb but not eutopic endometrium (samples obtained during the menstrual cycle) (Barrier et al., 2006a, b). However, in a study by Kawashima et al. (2009), the presence of HLA-G was observed using the MEM-G/1 mAb in eutopic endometrium but only in the menstrual phase. Furthermore, the presence of HLA-G has been reported in human decidual stromal cells (Blanco et al., 2008).

A striking observation is that the 5A6G7 and 2A12 mAbs stain the endometrial epithelial cells, whereas the MEM-G/1 and MEM-G/2 mAbs do not. And in the male reproductive tissues in general, the same pattern is observed, with no positive staining with MEM-G/1 and MEM-G/2 at all. The results presented here taken together with the study by Langat et al. of prostate tissue clearly indicate that HLA-G5 is the isoform present in the male reproductive system (Langat et al., 2006). This would further indicate that it is HLA-G5 and/or HLA-G6, which is located in the endometrial epithelial cells and that the MEM-G/1 and MEM-G/2 may fail to detect HLA-G5 when used in immunohistochemistry. The specificities of the MEM-G/1 and MEM-G/2 have been addressed in a publication by Menier et al. (2003). It is shown that MEM-G/1 and MEM-G/2 can detect all denatured isoforms of HLA-G in western blotting. However, in the case of immunohistochemistry, it is only shown that MEM-G/1 stains extravillous trophoblast cells in paraffin-embedded sections of human first-trimester placenta tissue. According to information from the manufacturer (Exbio Praha) both MEM-G/1 and MEM-G/2 stain paraffin-embedded trophoblast cells but other tissues with weak HLA-G expression are stained only with MEM-G/2. Thus, tissues with low expression or occurrence of HLA-G could perhaps show reduced or lacking reactivity with the HLA-G heavy chain specific antibodies. Furthermore, differences in fixation might also influence the binding of the MEM-G/1 and MEM-G/2 mAbs, however, the same procedure has been used for all tissues and it performs very well in placenta sections.

Some of the interesting results obtained from the immunohistochemical staining were the detection of HLA-G in normal testis, epididymis, and the weak staining in prostate tissue. The positive reaction pattern in testis and epididymis were shown by the use of 5A6G7 and 2A12 mAbs specific for the HLA-G5 and HLA-G6 soluble isoforms. Only 5A6G7 showed a weak positive staining in prostate tissue, but not 2A12. This difference resembles the difference observed regarding the staining pattern of extravillous trophoblast and endometrial epithelial cells. Again, the specificity of the two mAbs, 2A12 and 5A6G7, may be different due to differences in the reactivity with HLA-G5 and -G6, respectively. However, this is very speculative, and to our knowledge, no systematic comparison of the staining patterns of these two mAbs has been published. As mentioned previously, it has been chosen only to rely on the results obtained with the well-described 5A6G7 mAb in this study. Previous studies by Langat et al. (2006) have shown the presence of HLA-G5 in prostate tissue; however, other HLA-G antibodies were used. In the current study, only a rather weak positive staining with the 5A6G7 mAb was observed supporting the presence of HLA-G5 and/or -G6 in prostate tissue. In the current study, only prostate tissue samples from cases with hyperplasia were investigated. Furthermore, the fact that completely different anti-HLA-G mAbs were used in the two studies could also explain differences in the staining patterns of the two sets of mAbs. Langat et al. (2006) concluded that HLA-G expression in adenocarcinomas from the prostate gland was either reduced or no longer detectable. Additional studies in normal prostate tissue are necessary for obtaining clear conclusions about the expression of HLA-G in prostate tissue. It cannot be fully excluded that the weak reaction with 5A6G7 could be due to non-specific reactivity against e.g. neurocrine cells. In summary, the study by Langat et al. (2006) of HLA-G expression in a normal human prostate and the results obtained in the current study strongly suggest that the prominent isoform of HLA-G expressed in the male reproductive system is the soluble HLA-G5 isofrom.

To further validate, the presence of HLA-G in human seminal plasma, we also investigated the presence of sHLA-G molecules in seminal plasma samples from six male donors using a specific sHLA-G assay. All of these samples were positive for sHLA-G in varying concentrations. This may also indicate that there might be individual differences in the expression level of HLA-G in the reproductive tissues from male to male influencing the immunohistochemical results. In further investigations, it would be interesting to analyze a large number of seminal plasma samples to further compare the different sHLA-G levels between individuals. Hypothetically, there could be a correlation between sHLA-G levels and the genotype of the male donor and it could be interesting to obtain more information about these aspects.

To clarify the possibility that leukocytes in the semen may be a source for sHLA-G, seminal samples from four donors were paraffin-embedded and sections stained with the anti-HLA-G mAbs.
and a leukocyte marker. The results were negative and indicate that leukocytes in the semen do not seem to contribute to sHLA-G in the seminal plasma (Fig. 6).

In accordance with results from the western blot, the immunohistochemistry, and the ELISA experiments, there are strong indications of HLA-G being expressed in parts of the male reproductive system. In the testsis, HLA-G might have a functional role serving as an immuno-suppressive factor, and thereby avoiding recognition of ‘self’ sperm cells, which are considered as autoantigens for the immune system. One of the functions of HLA-G might be the maintenance of the testes as an immune privileged site. In this regard, it is interesting that Sertoli cells seem to be immunoprotective and they seem to locate HLA-G (Mital et al., 2009).

Several observations indicate that specific factors in seminal plasma can interact with cells and functional pathways in the female reproductive tract. It may lead to a range of cellular and molecular changes resembling an inflammatory response. The female genital tract is an immuno-competent mucosal site (reviewed in Robertson et al., 2003; Robertson, 2005). Seminal plasma is not mandatory for initiation of viable pregnancies since these occur using washed ejaculated sperm in artificial insemination. However, repeated exposure to semen in both animal models and in humans improve reproductive success (Robertson et al., 2003; Robertson, 2005). In mice, it seems that uterine ‘priming’ with semen can promote implantation and fetal growth in subsequent pregnancies, even in a partner-specific manner (Beer et al., 1975). In humans, live birth rates in couples undergoing IVF are significantly improved, when women are exposed to semen at the time of embryo transfer (Tremellen et al., 2000). Local effects of natural insemination in peri-ovulatory women involve infiltration of macrophages, dendritic cells, granulocytes and lymphocytes into the cervical epithelium and stroma shortly following intercourse; however, this inflammatory response was not observed following condom-protected intercourse (Robertson, 2005). These activated female immune cells may express cytokines and growth factors. Several factors in seminal plasma may be involved in the modulation of the inflammatory response in the cervix and the uterus. Two candidates for induction of tolerance to seminal antigens are transforming growth factor beta and prostaglandin E2, which can interact with cells and functional pathways in the female reproductive system. HLA-G being expressed in parts of the male reproductive system. In conclusion, the results of this study provide evidence that sHLA-G is present in varying amounts in human seminal plasma, and also that it is present in the testis and epididymis of the male reproductive system.

**Authors’ roles**

T.V.H. and M.H.L. designed the study and wrote the manuscript; M.B., M.H.L., M.B.P., M.W.N. and S.G.S. performed the experiments and prepared the data; M.H.L., M.B., T.V.H., L.G.L. and A.L. analyzed the data; and M.B., M.H.L. and S.G.S. coordinated and handled the collection of sample material.

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