

Antigens HLA-G, sHLA- G and sHLA- class I in reproductive failure

Olimpia Sipak-Szmigiel¹, Elżbieta Ronin-Walknowska¹, Cezary Cybulski²,
Tomasz Płonka¹, Jan Lubiński²

¹Chair for Obstetrics and Gynecology, Department for Feto-Maternal Medicine, Pomeranian Medical University, Szczecin, Poland

²Hereditary Cancer Center, Department of Genetics and Pathology, Pomeranian Medical University, Szczecin, Poland

Abstract: It can be supposed that relation between HLA-G polymorphism and sHLA-G protein expression are associated with successful embryo implantation and pregnancy maintenance. The aim of the study was the estimation specific differences in expression of sHLA-G and sHLA- class I antigens in women with reproductive failure in comparison with fertile women. The study sample enrolled 80 women, divided into 2 groups. The study group (B) enrolled 60 women with reproductive failure including 20 women with 3 recurrent spontaneous abortions in the first trimester of pregnancy (RSA), 20 women with empty sac (ES) and 20 women with 3 consecutive *in vitro* fertilization failures (IVFf). The control group (C) enrolled 20 fertile women with at least 2 children. Soluble HLA- class I antigens (sHLA-I) and soluble HLA-G (sHLA-G) were determined using ELISA test kits from IBio Vendor Laboratory Medicine, Inc. HLA-G allele found in individuals in our study were identified by comparing the obtained bp sequences of exon 2., 3. and 4. with bp sequences of HLA-G antigen published at the Nolan Research Institute website. The highest concentration of sHLA-I is noted among women with HLA-G 10401 allele which differed significantly for the mean sHLA-I concentration calculated for all the remaining alleles ($p < 0.0001$). The most prevalent alleles were: HLA-G 10101, 10102 and 10108 with sHLA-I concentrations among women bearing those alleles significantly lower in comparison to the HLA-G 10401 carriers ($p < 0.001$). Allele 10101 and 10102 was related to the lower significantly plasma sHLA-I concentrations than 10108 allele ($p < 0.02$). Lowest mean sHLA-G values were observed in the IVFf group with significant difference from the remaining groups ($p < 0.05$). To conclude, sHLA-G molecules is associated to certain HLA-G alleles and imply that sHLA-G levels are under genetic control. Low concentration sHLA-G seems to be prognostically important in IVF failure.

Key words: sHLA-G - sHLA-I - HLA-G polymorphism - Reproductive failure

Introduction

Detection of HLA-G by Gerathy *et al.* [9] in 1987 has begun further study on role of its expression, and function of non-classical Ib class antigens. Thorough investigation of HLA-G antigen expression in pregnancy at maternal-placental interface [5,19,22] and its influence on maintenance of maternal immune tolerance of the for semiallogenic embryo was initiated [1]. HLA class I and II antigens (HLA-A, -B, -C, -DR, -DQ and -DP), which not only play the key role in

transplantology but are also associated with various autoimmune disorders, were described in detail [4,32].

Non-classical HLA class I antigens (HLA-E, -F and -G), particularly HLA-G became the target of research regarding autoimmune response by inhibition of cytotoxic T cells (CTL) and NK cells function [21]. It is known, that HLA-G antigen gene is localized at the short arm of chromosome 6, coding for two forms of protein: cell membrane-liked and soluble [20,28]. It has been also proven, that soluble protein sHLA-G is present in maternal serum during pregnancy [19], in the placental blood and amniotic fluid [7,28]. Clinical research have indicated that certain immunologic situations significantly influence plasma sHLA concentration [24].

During pregnancy the following changes in sHLA-I plasma concentration were observed: significant rise

Correspondence: O. Sipak-Szmigiel, Dept. for Feto-Maternal Medicine, Pomeranian Medical University in Szczecin, Unii Lubelskiej Str. 1, 71-252 Szczecin, Poland; tel.: (+4891) 4253292, fax.: (+4891) 4253306, e-mail: olimpiasipak-szmigiel@wp.pl

in first and second trimester, with subsequent marked decrease in the third and during labor. sHLA-I levels during delivery were lower in comparison to the maternal blood [16]. Rebmann *et al.* [27] noted that both in placental blood and amniotic fluid sHLA concentrations were lower than in maternal blood. Our own study carried out in the group of women with high-risk pregnancy (APS, IUGR, PE) increased levels of sHLA-I were positively correlated with IFN gamma levels [30].

It was observed that variance in expression of sHLA-G proteins is associated with HLA-G alleles with sHLA-G levels being genetically controlled thus influencing function of immune regulation on various levels by sole existence of alleles responsible for "high" and "low" soluble form secretion [28].

Pfeifer *et al.* [24] have observed increased risk of idiopathic recurrent spontaneous abortions after IVF among women with low concentration of sHLA-G in peripheral blood in comparison to women with high levels this soluble antigen.

The aim of work is assessment of association between of soluble HLA-G and HLA-I concentration with HLA-G alleles and genotypes among women with spontaneous abortions in the first trimester of pregnancy in comparison to fertile women.

Materials and methods

Patients. Eighty women enrolled into the study were divided into two groups. The study group (S) comprising of sixty individuals with pregnancy failures included twenty women with three spontaneous abortions in the first trimester of pregnancy (RSA), twenty women with blighted ovum - "empty sac" (ES) and twenty women with triple IVF failures. The control group (C) comprised of twenty fertile women, who gave birth to at least two children in the past. In the group with pregnancy failures the following concurrent factors were excluded: karyotype abnormalities, hormonal disturbances, anatomical uterine malformations, infectious agents and autoimmune disorders [anti-cardiolipin antibodies (ACLA) and antinuclear antibodies (ANA) were tested].

sHLA I concentration in peripheral blood. sHLA class I were detected by ELISA method. Maxisorb plates (NUNC, Wiesbaden, Germany) coated overnight in 4°C with 100 µl with rabbit anti-mouse IgG antibody (DAKO, Zurich, Switzerland) (1:500 dilution) were subsequently washed three times with 300 µl of TWEEN20/PBS 0,02% solution (PBST) and incubated for one hour in the room temperature with 100 µl a-HLA I W6/32 serum (DAKO, Zurich, Switzerland) (1:500 dilution). Another washing with PBST was performed, the blocking with 300 µl of 3% BSA (Amersham, Pittsburgh, USA) for one hour in the room temperature was performed.

The plates were washed with PBST, and 100 µl of standard serum (courtesy of **) or of analyzed serum concentration was added and incubated in the room temperature for one hour. In the next step washing with 300 l of PBST (5x) was performed with subsequent incubation with 100 µl of peroxidase conjugated anti-2microglobulin (DAKO, Zurich, Switzerland). Following the incubation intensive washing was done (10×300 µl of PBST). The reaction was blocked after 15 minutes with 0,5 M H₂SO₄. Absorbance was measured at =490 nm ref=630 nm with BIO-

TEK microplate reader (BIO-TEK Instruments Inc, Vermont, USA).

sHLA-G concentration in peripheral blood. The analysis was performed with ELISA method with kits purchased from Bio Vendor Laboratory Medicine, Inc (catalogue no. RD194070100). Analytical sensitivity - 2 Units/ml.

Standard and analysed serums were added to the microplate wells, previously coated with mouse monoclonal a-sHLA-G antibody. Following one-hour incubation in the room temperature sHLA-G in the serum links with monoclonal antibody. Free antibody is washed-out in automatic washer with washing buffer. To all wells the conjugate is added (mouse monoclonal antibody (enzyme conjugated a-human β2-mikroglobulin). Subsequent incubation for one hour in the room temperature is performed. Excess of the conjugate is washed out with the washing buffer. Having added the enzyme substrate color reaction is performed with intensity relative to the sHLA-G concentration in the analysed samples. The reaction is stopped after 10 minutes of incubation with absorbance being measured with the reader at wavelength λ=450 nm. Standard curve is used to establish the concentration of proteins in the analyzed samples.

HLA-G typing. In all patients direct sequencing of exons 2,3 and 4 of HLA-G gene was performed. Genomic DNA was isolated from the peripheral blood leukocytes by purification with phenol/chloroform and precipitation with ethanol. DNA amplification was performed using polymerase chain reaction with primers and conditions as described previously [23]. After purification in Microcon-100 columns (Amicon) PCR products were sequenced with the same primers, using fluorescently labelled dideoxy chain terminators from an ABI Prism v 3.0 kit (Applied Biosystems) in an ABI 377 automated sequencer. ABI PRISM 377 Collection Software and Sequencing Analysis Software version 3.0 were used for data collection and analysis.

Definition of HLA-G allele. HLA-G alleles were identified by comparison of the sequences of exon 2, 3 and 4 in the patients with the sequences of HLA-G alleles published at the Nolan Research Institute website.

Statistical analysis. sHLA- I and sHLA-G concentrations were described by ± standard error of the mean (SEM). To analyze statistical differences between continuous variables for different groups the U Mann-Whitey test corrected for group size. Kolmogorov-Smirnov test was used to establish sHLA-G concentration ranges which would most appropriately differentiate between the groups.

Non-continuous variables were described by the number and prevalence. To analyze statistical correlations between non-continuous variables Pearson's Chi-square test, Fisher's exact test and logistic regression model were used. P values of p<0,05 were considered significant in all performed tests. Borderline significance was defined as p>0,05 and p<0,10. STATISTICA-6,0 and STATA-5,0 were used for statistical analyses.

Ethical issues. Consent to the experiments was obtained from the Ethical Board for Animal Experimentation PAM- BN- 001/131/02

Results

As shown in the Table 1 the highest concentration of sHLA-I is noted among women with HLA-G 10401 allele (513,4 89,5 SEM ng/ml) which differed significantly for the mean sHLA-I concentration calculated for all the remaining alleles (p<0.0001). The most

Table 1. sHLA I and sHLA G concentrations (mean SEM) correlated with alleles in the studied group.

Allele	N	s HLA I [ng/ml]		p*	s HLA G [U/ml]		p*
		Mean	SEM		Mean	SEM	
10101	51	157,9 ^{a, b}	9,5	0,067	17,26	3,29	Ns
10102	52	180,6 ^{a, b}	15,5	0,093	20,59	6,80	
10103	2	327,5	149,5		2,00		
10106	5	165,0 ^a	37,2		11,60	7,54	
10108	28	222,6 ^a	23,3	0,055	19,77	9,31	
10109	1	148,0			2,00		
10401	7	513,4	89,5	0,0001	6,93	3,12	
103	2	141,0			7,00		
106	6	166,7 ^a	22,6		4,73	1,44	
Razem	154	196,0	10,4		17,27	3,06	

^asHLA I concentrations lower in comparison to the ones noted for 10401 allele with $p < 0,001$; ^bsHLA I concentrations lower in comparison to the ones noted for 10108 allele with $p < 0,02$; p* - statistical significance with the analyzed allele in comparison to the remaining alleles

prevalent alleles were: HLA-G 10101, 10102 and 10108 with sHLA-I concentrations among women bearing those alleles significantly lower in comparison to the HLA-G 10401 carriers ($p < 0,001$). Allele 10101 and 10102 was related to the lower plasma sHLA-I concentrations than 10108 allele ($p < 0,02$). The highest concentrations of sHLA-G was found among HLA-G 10102, 10101 and 10108 allele carriers, however with no statistical differences noted between the alleles.

Table 2 indicates that the highest mean values for sHLA-I was noted in BO group ($252,3 \pm 43,8$ SEM U/ml); No differences noted in mean concentration of sHLA-I between the groups. Lowest mean sHLA-G values were observed in the IVFf group ($3,32 \pm 0,90$ SEM ng/ml) with significant difference from the remaining groups ($p < 0,05$).

Discussion

In spite of the extensive research regarding possible role of both membrane-linked and soluble forms of HLA-G at the maternal-placental interface no conclusive relation with pregnancy outcome was elucidated so far. Moreover, its role in the blastocyst implantation and IVF success remains unclear.

It is suspected that soluble forms of HLA-G are probably transported from the mononuclear peripheral blood cells containing HLA-G mRNA [13,10]. While the most important source for sHLA-I proteins are liver and lymphocytes [2]. Soluble HLA-G was mostly found in the syncytiotrophoblast and other trophoblastic cells [15,17,19].

Expression of soluble HLA-G is related to the Fas/FasL related CD4+ and CD 8+ T-lymphocyte apoptosis [3,6,11]. HLA-G1 ad soluble isoform HLA-G5 protects from lysis by Tc lymphocytes. Of the sol-

uble HLA-G isoforms present in serum during pregnancy the main one is sHLA-G2, which does not bind $\beta 2m$. Isoform sHLA-G1 includes $\alpha 2$ domain necessary to bind $\beta 2m$ and peptides between light and heavy chains while sHLA-G2 does not include $\alpha 2$ domain and binds itself into HLA homodimers similar to class II, which do not recognize $\beta 2m$ but may bind peptides [12].

sHLA-G presence in culture supernatants from IVF is linked with high degree of success in the procedure [15,29]. Other studies where failures to implant in the situation of sHLA-G absence in the pre-implantation supernatants from embryonic cultures support this thesis [8,23,32].

Rizzo *et al.* [30] suggest a role for sHLA-G molecules in the ovulatory process and propose the follicular fluids (FFs) analysis for sHLA-G molecule presence as a useful tool oocyte selection in IVF.

In our study we found that the highest concentration of sHLA-I was noted among women with sHLA-

Table 2. Mean (\pm SEM) concentrations of sHLA I and sHLA-G in each of the subgroups among women with pregnancy failures (BO, RSA, IVFf,) and in the control group (C)

Groups	N	s HLA I[ng/ml]		s HLA G [U/ml]	
		Mean	SEM	Mean	SEM
BO	20	252,3	43,8	14,26 ^a	5,05
RSA	20	189,0	21,1	18,80 ^a	4,57
IVFf	20	171,8	19,1	3,32	0,90
C	20	180,1	23,7	34,17 ^a	15,10
Total	80	198,3	14,5	17,64	4,26

^alowest sHLA-G concentrations in comparison to the IVFf group with $p < 0,05$ (U Mann-Whitney test)

G 10401 allele (513.4 ± 89.5 SEM ng/ml) and differed significantly from mean sHLA-I concentrations calculated for all the remaining alleles ($p < 0.0001$). The most prevalent HLA-G alleles were 10101, 10102 and 10108 related to significantly lower mean sHLA-I values in comparison to HLA-G allele 10401 ($p < 0.001$). Among HLA-G allele 10101 and 10102 carriers significantly lower levels of sHLA-I in analysed samples were noted than among 10108 allele carriers ($p < 0.02$). We have observed that the highest sHLA-G mean concentrations were observed among HLA-G 10102, 10101 and 10108 carriers, with insignificant concentration differences related to each of these alleles.

In our research the highest values of mean sHLA-I concentration was found in the BO group (252.3 ± 43.8 SEM U/ml); No significant difference in mean sHLA-I concentrations between the groups was noted. The lowest mean sHLA-G levels were observed in the IVF group (3.32 ± 0.90 SEM ng/ml) differing significantly from the remaining groups ($p < 0.05$).

Differently from the results obtained in our study, Rebmann *et al.* [28] have found that among people bearing HLA-G 01013 allele (8.1 ± 1.7 SEM ng/ml) or "null" allele HLA-G 0105N (8.2 ± 3.2 SEM ng/ml) significantly lower serum levels of sHLA-G in comparison to people bearing other alleles were noted, while individuals with HLA-G 01041 allele tended to present significantly higher sHLA-G levels (42.5 ± 4.6 SEM ng/ml), which may indicate genetic control over sHLA-G secretion on various levels of immune regulation by existence of HLA-G alleles responsible for "high" and "low" secretion of soluble forms [28]. In the previous studies Rebmann *et al.* [27] have proven that mean sHLA-G concentrations did not differ between men (24.9 ± 3.0 SEM ng/ml) and women (20.1 ± 2.1 SEM ng/ml), but among HLA-A11 carriers sHLA-G concentrations (13.0 ± 2.0 SEM ng/ml) were significantly ($p < 0.001$) lower than among other HLA-A allele carriers. These researchers have also observed that sHLA-G levels during labor did not differ significantly from the control group, while being significantly ($p < 0.001$) lower in placental blood (13.8 ± 1.5 SEM ng/ml). Mean sHLA-G values (15.5 ± 1.0 SEM ng/ml) in amniotic fluid were significantly ($p < 0.001$) lower than in plasma, while individual sHLA-G levels did not correlate with sHLA-I levels [27].

Hunt *et al.* [12] while assessing sHLA-G levels in pregnancy have observed that these concentration increase in the first trimester of pregnancy reaching plateau in the second and third trimester, with moderate decrease at the end of pregnancy. Other researcher when studying sHLA-G concentrations among pregnant women and non-pregnant did not observe significant differences [14,24,26].

Implantation of embryo in the procedure of IVF remains a difficult process, as 62% of oocytes may be

rejected after 12th week of pregnancy with key role for fetus survival is also played by sHLA-G2 [8].

Lately, many researchers have been investigating the expression of this molecule, especially sHLA-G in the supernatants from oocyte culture, and its influence on embryo survival following the transfer [31,35].

Van Lierop *et al.* [34] during research into human embryos culture in 8 cell morula before formation of blastocyst did not detect sHLA-G.

Noci *et al.* [23] have shown the presence of sHLA-G in 36.2% of single embryo cultures after 72 hours, while in 8.9% of cultures sHLA-G was detected after 48 hours. Sher *et al.* [32] while investigating 594 embryos from 201 women observed presence of sHLA-G among at least one embryo from 65% of women.

Pfeiffer *et al.* [24] have found that women with IVF failure manifested by spontaneous abortion in the early pregnancy presented lower sHLA-G and sHLA-I levels in the pre-ovulation period and during pregnancy compared to women with normal pregnancy.

In pregnancy failures manifesting as blighted ovum - "empty sac" (ES) concentrations of soluble HLA-I and sHLA-G were not investigated so far nor was the association between the concentration and HLA-G polymorphisms.

sHLA-G molecules is associated to certain HLA-G alleles and imply that sHLA-G levels are under genetic control. Low concentration sHLA-G seems to be prognostically important in IVF failure.

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