In Vitro Fertilized Embryos do not Secrete Detectable HLA-G on Day Two

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ABSTRACT

Background: Pregnancy is a successful transplantation. The factors evading rejection of the fetus are poorly understood. Recently an interest has grown, in HLA-G molecules as one of these factors. Since these antigens are mainly expressed on the surface of cytotrophoblasts that are in direct contact with maternal tissues, it has been suggested that these molecules may have a role in induction of immune tolerance in mothers. **Objective:** to find the association of soluble HLA-G (sHLA-G) and the success of pregnancy with intracytoplasmic sperm injection (ICSI) procedure. **Methods:** In this study, the supernatant of 287 individually cultured embryos corresponding to 96 women under ICSI procedure were assayed for soluble HLA-G1 and G5 by a sandwich ELISA. **Results:** Clinical pregnancy successfully occurred in 30 of candidates. No differences in clinical parameters (age, infertility duration, stimulation regimen) were observed between pregnant and nonpregnant women under ICSI procedure. None of the embryo supernatants in either group showed any detectable sHLA-G molecules. **Conclusion:** Our results showed that detectable level of sHLA-G is not produced by day 2 embryos and such a measurement may not provide reliable information for embryo selection and estimation of pregnancy success.

Keywords: HLA-G, Pregnancy, Intracytoplasmic Sperm Injection

INTRODUCTION

Implantation rates following in vitro fertilization (IVF) have not improved significantly during the last decade. Embryo selection for transfer must be based not only on quality criteria but on several molecules involved in the regulation of growth and development of embryos. The absence of rejection by the maternal immune system is an additional prerequisite for a successful pregnancy.

The lack of classical HLA class I molecules on cytotrophoblast cells is necessary to prevent allorecognition by maternal cytotoxic T lymphocytes that induce activation of NK cells. HLA-G may be a factor inhibiting NK cells and preventing fetus rejection by maternal immune system. HLA-G is one of the histocompatibility antigens with both

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maternal and embryonic sources. This protein is detectable by immunohistochemistry procedures on some cells mainly on cytotrophoblast cells and to a lesser extent on thymus, cornea and erythroid cells (1,2). Soluble HLA-G molecules have been studied in plasma, serum, amniotic fluids, embryo culture supernatants, cell culture supernatants from placenta, follicular fluids and cord blood (1,2,3).

HLA-G was found to be involved in cellular adhesion, thus suggesting that it might play a role in blastocyst attachment to the uterine epithelium. Adherence of blastocysts may signal the trophectoderm differentiation to cytotrophoblast which invades the maternal decidua and spiral arteries, resulting in vascular remodeling. Furthermore HLA-G as a modulator of angiogenesis, via remodeling of uterine arteries, may ensure an adequate supply of nutrients and oxygen to the fetus (4). The cytotrophoblast is also in contact with various effector cells of the maternal immune system. Interaction of HLA-G with the maternal immune cells has been proposed to have a crucial role in inducing local tolerance at the site of implantation (1,2,5).

HLA-G involvement during pregnancy has a novel role as a tissue protective molecule during chronic cutaneous inflammatory disease (1,6). An altered expression of HLA-G may play a role in some of the complications of pregnancies for example pre-eclampsia and recurrent spontaneous abortion (7,8).

Alternative splicing of the HLA-G gene results in seven different transcriptional isoforms (1,2), four of which encode potential membrane bound products (G1-G4), whereas three others encode soluble proteins (G5-G7). HLA-G molecules are recognized by various antibodies. Total HLA-G is detectable by a monoclonal Ab toward the alpha-1 domain that is common to all HLA-G isoforms. HLA-G1 and soluble HLA-G5 have a complete structure. A mAb toward beta 2 microglobulin chain associated with alpha chain detects HLA-G1 and G5. Due to the presence of a stop codon in intron 4, soluble HLA-G5 and G6, yield an open reading frame encoding a peptide with 21-amino acids. The mAbs directed toward peptides unique to intron 4 specifically detect soluble HLA-G5 and G6. HLA-G1 might contribute to the soluble pool by shedding or cleavage. Although soluble HLA-G can be easily evaluated, the cellular source of secretion is controversially debated.

It has been suggested that the identification of soluble HLA-G in human embryo culture media could demonstrate developmental potential of the embryo and it could serve as an additional marker for selecting those embryos most likely to implant (9). Therefore, this study was designed to measure the level of soluble HLA-G in embryo cultures on the second day following ICSI.

MATERIALS AND METHODS

Patients and Intra Cytoplasmic Sperm Injection (ICSI). Ninety–six infertile couples with the “male factor” being the prominent cause of infertility participated in this study. All patients used their own eggs and had a normal uterine cavity and endometrial thickness. The patients were included in this study independent of their age, prior number of ICSI cycles and causes of infertility. ICSI was carried out for only one cycle per couple at one of the IVF clinics in Rasht, Iran (Family hospital). The proposal was approved by Ethical Committee of Guilan University of Medical Sciences.

After pretreatment on the 21st day of the cycle, ultrasound examination was performed for all women to exclude those with ovarian cysts > 15 mm or those with any fibroma, polyp, severe hydrosalpinx and endometrial hyperplasia. All patients were stimulated with a long protocol using subcutaneous injection of GnRh agonist (Suprefact), 0.5 ml per day. Plasma oestradiol concentrations and ovarian cysts were checked after the beginning of menstruation. Baseline oestradiol concentrations of <50 mg/ml and ultrasounds showing no ovarian cysts >15 mm diameter were required before initiation of HMG (Human Menopausal Gonadotropin) therapy. HMG (Merional) was injected subcutaneously at doses of 450-750 IU per day and the dose of GnRh agonist decreased to 0.2 ml per day. Vaginal ultrasound examination was performed during the period to control the growth of follicles. 10,000 IU of human chorionic gonadotropin (HCG) was then administered when at least 3 follicles with a mean diameter of 18 mm were achieved. Oocytes were retrieved by aspiration of follicles under ultrasound guidance and general anesthesia 36 hrs after HCG administration. Progesterone, 50 mg per day, was administrated from the day of oocyte retrieval.

Collected oocytes were cultured for 2-4 hours at 37° C with 6% CO2, and 90% humidity. All media were obtained from G series (Vitrolife, Sweden). Cumulus oocyte complex was denuded in 1% hyaluronidase drops for less than 1 minute. Standard ICSI procedure (10) was performed 4-6 hrs after oocyte retrieval using an Olympus inverted microscope (IX70) and Navishige micromanipulator in the G series buffered medium. Oocytes were then cultured individually in 50 µl drops under an oil overlay inside the incubator. Fertilization was checked 15-21 hrs after ICSI. Embryo qualities were evaluated as to cell number, fragmentation and regularity of blastomeres as described in Table 1 (9). After 48 hrs, 3-5 embryos were chosen according to the morphological criteria for transfer under ultrasound guidance using an embryo transfer catheter (Labotect, Germany). The remaining culture supernatants of embryos were carefully transferred to a fresh tube and stored at -20° C until used.

Table 1. Morphological evaluation criteria employed for embryo quality grading

			Score
No. of Blastomeres	Day 2 (48 h) Day 3 (72 h)	≥ 4 ≥ 6	0
	Day 2 (48h) Day 3 (72h)	2-3 4-5	1
	Day 2 (48h) Day 3 (72h)	< 2 < 4	2
Size of Blastomeres	Regular		0
	Irregular		1
Fragmentation of embryos (%)	0-10 11-20 21-50 >50		0 1 2 3
			Total score
Embryo quality	Type A Type B Type C Type D	Very good Good Poor poor	0-1 2-3 4-5 > 5

The dose of progesterone was either increased to 100 mg per day or 2 suppository cyclogest 400 were administrated after the transfer of embryos. A single serum beta HCG test was performed 14 days after embryo transfer. Patients with a positive HCG test received the same hormonal support of the ongoing pregnancy. A clinical pregnancy was confirmed by transvaginal ultrasonography when an intrauterine gestational sac and a fetal heartbeat were detected after the seventh week of gestation.

Soluble HLA-G Determination. Soluble HLA-G levels of individual embryo culture supernatants of the 2nd day (42-48 hrs) cultures were determined with a new commercial double monoclonal Ab sandwich immunoassay kit (Exbio/BioVendor, Czech Republic). In this ELISA kit in the first layer the plate was coated with a monoclonal Ab toward the alpha chain associated with β2m (MEM-G9). After adding the sample, another mAb toward β2m chains (W6/32) conjugated with HRP was used in the second layer as a detecting Ab. These two monoclonal antibodies can recognize shedding HLA-G1 of the embryo and the soluble HLA-G5 that the embryo secretes. Lyophilized sHLA-G standard (1000 IU/ml) was provided for preparation of the assay standards. The standards were run in duplicate but duplication of the test was not possible due to the limited sample volume. The sensitivity of the assay was 1 IU/ml (according to the producers' description) that is equivalent to 0.4-0.5 ng/ml.

RESULTS

Table 2 compares the clinical characteristics of pregnant and nonpregnant women who have participated in this study. Table 3 shows the correlation between clinical pregnancy and embryo quality. The number of blastomeres, regularity or irregularity of blastomeres and fragmentation percentages defined embryo quality. No significant differences in clinical characteristics (female age, infertility duration, and stimulation regimen) were found between pregnant and nonpregnant women. However, the total number of oocytes collected, inseminated and fertilized in pregnant women was significantly higher than those in nonpregnant women ($p<0.01$ and $p<0.0001$, respectively).

Table 2. Evaluation of clinical parameters in pregnant and non-pregnant women

	Pregnancy (n=30)	No pregnancy (n=66)
Female age (years)	30 ± 5.2	32.4 ± 6.7
Infertility duration (years)	7.1 ± 4.8	7.2 ± 4.8
No of ampoules	27.2 ± 5.3	27.7 ± 7.2
Duration of stimulation (days)	12. 2 ± 2.7	12.60 ± 1.9
Retrieved oocytes	9.8 ± 5.6	7.5 ± 5.6
Inseminated oocytes[a]	8.4 ± 4.2	6.03 ± 4.1
Fertilized oocytes[b]	5.9 ± 3	3.36 ± 2.3
Fertilization rate (%)	70.1 ± 20.1	64.07 ± 25.9
Transferred embryos[b]	3.7 ± 1.2	2.5 ± 1.4
No of blastomers	3.8 ± 0.97	3.69 ± 0.92

Values are means ± SD. Significant differences were found with the Student's t-test ([a] $p<0.01$, [b] $p<0.0001$)

Fertilization rate showed no significant differences between groups. The total number of transferred embryos showed significant increase in pregnant women when analyzed with t-test ($P<0.0001$). A significant Correlation was found between embryo quality and the presence of pregnancy ($P<0.01$; Yates corrected χ^2).

Table 3. Correlation between embryo quality and the clinical pregnancy

Clinical pregnancy / Embryo quality	Pregnancy No. of samples (%)	No pregnancy No. of samples (%)	Total embryos No. (%)
A*	99 (88)	129 (74)	228 (79)
B*	14 (12)	39 (22)	53 (19)
C	0	6 (4)	6 (2)
D	0	0	0

*Significant differences were found with Yates corrected χ^2 test, P<0.01.

Two hundred and eighty seven day-2 embryo culture supernatants from 96 patients were assayed. Soluble HLA-G1 and G5 were not detected in any of the day-2 culture media. Thirty patients had successful pregnancy which resulted in the pregnancy rate of 31.3% (30/96).

DISCUSSION

Soluble HLA-G was not found in any of the day 2 (42-48 hrs) culture media droplets of embryos that were selected for transfer. This is similar to the findings of Van Lierpot et al. (11) and Sageshima et al (12,13). However other researchers have found sHLA-G in day-2 culture media and suggested that its presence improves the success rate of *in vitro* fertilization embryo transfer and limits multiple pregnancy rates (14,15,16). There is, however, conflicting evidence produced by other studies which found no sHLA-G in the media of trophoblasts but found HLA-G1 fragments shed from the cell surface (17). In relation to these findings, the data on the presence of sHLA-G protein does not match with the mRNA studies. A study has reported that HLA-G5 mRNA was only expressed by 20% of morulae and blastocyts and was not detected in 2- to 8- cell embryos suggesting that the presence of sHLA-G in media may be due to HLA-G protein remaining from maternal oocyte stores produced before embryonic genomic activation and raised doubt about the clinical value of sHLA-G for evaluation of embryo quality (18). Other investigators did not find any mRNA for HLA-G in human 2 to 8 cell embryos or in blastocytes (19). Such results, that two to eight cell embryos did not have sHLA-G mRNA, supported our findings. A review article (20) concluded that the analysis of sHLA-G in the follicular fluid might be a reliable tool in oocyte selection.

Considering the possible importance of the stages of embryo development in the production of sHLA-G, we also examined the day 3 culture media. sHLA-G or perhaps shedding HLA-G1 was detected on day 3 embryo culture supernatants. However, the numbers of positive specimens were not significant and require further studies. Accordingly, several studies have indicated the presence of sHLA-G in day 3 embryo cultures (14,16,21,22).

Different HLA-G alleles affect the levels of the soluble forms of the protein that is produced (23). The genotype of Iranian population for null HLA-G allele has been recently published and is reported to be as high as 20% (24). The presence of null allele is associated with the instability of HLA-G1 and G5 proteins, therefore the great presence of this gene in the women participating in our study could be the reason for lower frequencies of positive results. However, other isoforms may substitute for HLA-G1 protein, therefore, its lack causes no problem in intact pregnancies (25,26).

Due to the source of sHLA-G, the immune status and the HLA-G alleles, the nature and the quality of sHLA-G molecules are variable in different individuals (3). Furthermore,

technical differences such as sensitivity, specificity and cross reactivity of the monoclonal antibodies and culture conditions in different ICSI procedures may affect the final results of these types of studies and should be taken into consideration (27,28). However, a highly sensitive standard ELISA may be helpful in providing comparable results.
Since sHLA-G in day-3 embryo cultures appears to be more detectable than in day-2 cultures we suggest a standard ELISA-based study to be performed at pictogram level on day-3 cultures. Studding the allelic frequency may also help with the analysis of the results. The study of HLA-G homologous in other species including murine Qa-2 (29) provides a more flexible condition where culture and transfer of individual embryos are possible and the effect of each HLA-G positive embryo on the outcome of pregnancy could be shown, but this may only be materialized when standard antibody against Qa-2 becomes available.

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