Secretion of soluble HLA-G by day 3 human embryos associated with higher pregnancy and implantation rates: assay of culture media using a new ELISA kit

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Abstract

Identification of chemical markers in human embryo culture media that relate to embryo quality and implantation potential could be an invaluable tool in embryo selection for transfer. Embryonic secretion of soluble human leukocyte antigen G (sHLA-G) has been postulated to be a marker for 'embryonic competence'. This study examines sHLA-G concentrations in day 3 culture media droplets of embryos that were selected for transfer, as well as those being cryopreserved. A total of 712 embryo culture supernatants from 83 patients were assayed. Soluble HLA-G was detected in 306 of the 712 samples tested. In the 58 transfers in which at least one embryo selected for embryo transfer was positive for sHLA-G, the pregnancy rate was 64% (37/58) and the implantation rate per embryo transferred was 38%. In contrast, patients receiving only embryos that were negative for sHLA-G had both a lower pregnancy rate of 36% (9/25; P < 0.05) and a decreased implantation rate (19%; P < 0.05). Expression of sHLA-G was also related to increasing cell stage. Concentration of sHLA-G in embryo culture media was variable and in the low range (3-10 ng/ml). These data suggest an association between implantation potential and embryonic secretion of sHLA-G. The commercial assay kit utilized allowed for same day assessment of sHLA-G secretion. Addition of sHLA-G status to traditional morphological criteria may be useful as a clinical tool for embryo selection.

Keywords: embryo culture, embryo marker, HLA-G, implantation, IVF, pregnancy

Introduction

The process of implantation requires the embryo to thwart the maternal immune system to avoid rejection. Soluble human leukocyte antigen G (sHLA-G) is present in many body fluids, and may confer immune tolerance to the embryo (Moreau et al., 1998; Hvid, 2004; Hunt et al., 2005). HLA-G expression has been detected in early preimplantation embryos (Juriscova et al., 1996a). It has been suggested that establishment of a successful pregnancy is contingent on embryonic expression of this factor (Juriscova, 1996b; Moreau et al., 1998; Fuzzi et al., 2002). Several recent studies have indicated a relationship between secretion of sHLA-G, high quality embryos and pregnancy outcome (Hviid et al., 2004; Sher et al., 2004, 2005a,b; Noci et al., 2005; Yie et al., 2005).

Identification of a chemical marker in human embryo culture media that relates to embryo quality and implantation potential could be an invaluable tool in conjunction with morphological parameters in embryo selection for transfer. The health risk for both patient and fetus associated with high order multiple pregnancy has spearheaded a movement to reduce the overall number of embryos being transferred to just two for young patients. Embryo selection based on critical assessment of morphological parameters and culture to the blastocyst stage have helped in moving towards this goal (Shoukir et
al., 1997; Desai et al., 2000; Fisch et al., 2001; Gardner and Sakkas, 2003; Neuber et al., 2003; Racowsky et al., 2003; Scott, 2003).

This study explores the possibility that sHLA-G secretion by human embryos may provide additional information regarding embryonic potential. Concentrations of sHLA-G were assessed in culture media supernatants at the time of embryo selection for transfer and retrospectively related to embryo quality and pregnancy outcome. The second goal was to see if a new commercially available ELISA kit could be used for detection of the low quantities of sHLA-G that might be secreted by individual embryos.

Materials and methods

Patients

Eighty-three women undergoing IVF at the Cleveland Clinic Fertility Centre at Beachwood, between March and August 2005, were involved in this study. Patients over 39 were not included in this initial trial, since data analysis would be complicated by the increased rate of aneuploidy with age and the lower overall pregnancy rates irrespective of embryo quality with this age group. No other patient selection or exclusion criteria were used.

Pregnancy testing was performed 16 days after embryo transfer. Clinical pregnancy was confirmed by the presence of a fetal heart on ultrasonic examination at 6–8 weeks. All outcome data are expressed in terms of clinical pregnancy and pregnancy outcome. The implantation rate is defined as the number of fetal hearts divided by the total number of embryos transferred.

Ovarian stimulation and transfer

Ovulation induction was carried out after down regulation with leuprolide acetate 1 mg (Lupron; TAP Pharmaceuticals, Lake Forest, IL, USA) starting at mid-luteal phase and then decreased to 0.5 mg at the initiation of FSH stimulation. Baseline oestradiol concentrations of <70 pg/ml and vaginal ultrasound showing no follicles >20 mm were required before initiation of FSH therapy. FSH (Follistim; Organon or Gonal F; Serono) was initiated at a dose of 225 IU per day unless previous stimulations indicated otherwise. When at least two follicles were of 18 mm mean diameter, 10,000 IU of human chorionic gonadotrophin (HCG) was administered 36 h before scheduled oocyte retrieval. Oocytes were recovered by transvaginal aspiration of follicles under ultrasound guidance. Embryo transfer was performed 3 days after the oocyte retrieval under ultrasound guidance using a Wallace SureView catheter.

Embryo culture

Patient oocytes were fertilized by intracytoplasmic sperm injection 3–4 h after retrieval. Fertilization check was performed the following morning. Zygotes were cultured individually in 20-µl medium drops under an oil overlay. Human tubal fluid medium (HTF; GenX) supplemented with 10% synthetic serum substitute (SSS; Irvine) was used for embryo culture until transfer on day 3. All culture was performed at 37°C with 5% CO₂ and air. Embryo selection for day 3 transfer was based solely on morphological parameters. Embryos were graded on the basis of cell number, regularity of blastomeres, good blastomere expansion, fragmentation level and signs of embryonic compaction. Transfer number was based primarily on patient age. Patients under 36 years generally received two embryos, unless poor embryo morphology was a concern. Patients between 36 and 39 years had two or three embryos, depending on embryo quality and the patient's ability to consider selective reduction in case of high order multiple pregnancy.

Good quality embryos not selected for transfer were cryopreserved on day 3 if they were between 6 and 8 cells with less than 20% fragmentation. All embryos not transferred or frozen on day 3 were moved to new dishes containing 20-µl drops of Global blastocyst medium (GenX) with 10% SSS and cultured for an additional 2–3 days. Embryo cleavage, compaction, morula and blastulation were monitored from day 3 to day 6 for the spare embryos kept in extended culture. The culture media drops from the day 3 dishes were collected and frozen at ~70°C for sHLA-G testing.

Assessment of sHLA-G

A new commercially available double monoclonal sandwich immunoassay kit for the quantitative measurement of s-HLA-G was utilized for assessment of embryo culture supernatants (Ex-Bio; Czech Republic). Lyophilized sHLA-G standard (1000 IU/ml) was provided for preparation of assay standards ranging from 3.125 to 200 IU/ml (conversion factor: 2 IU/ml equivalent to 1 ng/ml according to product literature). Culture media samples, negative control media and sHLA-G standards were incubated in 96-well plates precoated by the manufacturer with mouse monoclonal anti-sHLA-G antibody. The negative control was HTF medium with 10% SSS. The standards and negative control media were run in duplicate. Embryo supernatants could not be run in duplicate due to small sample volume. Upon collection all sample volumes were normalized before adding to the microtitre plate. After 1 h at room temperature, wells were rinsed three times with wash solution. Mouse monoclonal antihuman β2-microglobulin antibody labelled with horseradish peroxidase (HRP) was added to each well. Reaction between this conjugate and the immobilized antibody sHLA-G complex bound to the wells was allowed for 1 h. Wells were once again rinsed three times. Substrate (H₂O₂ with tetramethylbenzidine) was added to each well to react with any bound HRP-conjugated antibody. Stop solution was added after 15 min. A Molecular Devices ELISA plate reader was used for spectrophotometric assay of absorbance at 450 nm. The sensitivity of the assay was 1 ng/ml (2 IU/ml).

Data analysis

Embryo morphology, disposition and corresponding HLA-G concentrations were tracked for each cultured embryo. Concentration of sHLA-G in day 3 culture medium supernatants was retrospectively analysed. To be considered positive, embryo culture samples had to have absorbances
above the negative media control. In addition, since the stated low-end detection limit of the assay was 2 IU/ml (1 ng/ml), media samples falling below this cut-off were not reported as positive. It was hoped to reduce false positives by setting these limits. Culture supernatants meeting both criteria were designated as 'positive'.

For data analysis, transfer cycles in which at least one embryo selected for replacement expressed sHLA-G were grouped as 'positive'. If none of the transferred embryos secreted sHLA-G, these cycles were categorized as 'negative'. Culture media of non-transferred embryos was also examined for the presence of sHLA-G. The relationship between sHLA-G status and final disposition of the embryo, i.e. day 3 freeze, day 5/6 blastocyst freeze or arrested/discarded, was also studied. Based on concentration of sHLA-G in culture media, embryos were classified as 'low secretors' (expressing 3–10 ng/ml) or 'high secretors' (>10 ng/ml). Statistical differences between groups were analysed using the Student's t-test and chi-squared analysis.

Results

A total of 712 embryo culture supernatants from 83 consecutive patients were assayed. Soluble HLA-G presence was detected in 309 of the 712 samples tested (43%).

The concentration of sHLA-G in day 3 embryo culture media was variable, ranging from 3 to 50 ng/ml (Figure 1), with the majority of samples falling in the low range (3–10 ng/ml). Higher concentrations of sHLA-G (>10 ng/ml) were detected in only 14% of transferred embryos. No association between the higher concentration of sHLA-G and pregnancy was noted. Soluble HLA-G was detected in day 3 culture media of 47% (114/245) of embryos selected for cryopreservation either on day 3 or on day 5 or 6, and 40% of the discarded/arrested embryos (112/277).

The overall pregnancy rate for the 83 patients examined during this study was 55% (46/83). Table 1 compares cycles in which at least one sHLA-G positive embryo was transferred to cycles where all transferred embryos were negative for sHLA-G. The average patient age was 32.3 ± 5.2 and the mean number of embryos being transferred was 2.29 ± 0.5. No significant differences were found in patient and cycle characteristics between the two groups.

Pregnancy and implantation rates for the sHLA-G positive and sHLA-G negative transfer groups are shown in Figure 2. In the 58 transfers where at least one of the embryos selected for embryo transfer was positive for sHLA-G, 64% of patients (37/58) successfully achieved a pregnancy. In the remaining 25 transfers, where none of the embryos selected secreted detectable amounts of sHLA-G, the pregnancy rate was only 36% (9/25; P < 0.05). The implantation rate in sHLA-G positive transfers was also significantly higher, 38% per embryo versus 19% in transfers where none of the embryos selected expressed this factor (P < 0.05).

Figure 3 depicts the relationship between secretion of sHLA-G and embryonic cell stage. Embryos were classified according to cell stage and the percentage of embryos that were sHLA-G positive was calculated for each group. Soluble HLA-G secretion ranged from 37 to 53% amongst the different cell stage groupings. The percentage of embryos secreting sHLA-G was significantly higher with increasing cell stage (P < 0.05).
Table 1. Comparison of cycles in which at least one sHLA-G positive embryo was transferred with cycles where all transferred embryos were negative for sHLA-G.

<table>
<thead>
<tr>
<th>Cycle characteristics</th>
<th>sHLA-G positive</th>
<th>sHLA-G negative</th>
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<tbody>
<tr>
<td>No. of embryos transferred</td>
<td>58</td>
<td>25</td>
</tr>
<tr>
<td>No. of previous cycles</td>
<td>1.40 ± 0.84</td>
<td>1.52 ± 0.87</td>
</tr>
<tr>
<td>Age of patient (years)</td>
<td>32.3 ± 5.8</td>
<td>32.4 ± 3.4</td>
</tr>
<tr>
<td>No. of embryos transferred</td>
<td>2.35 ± 0.51</td>
<td>2.16 ± 0.37</td>
</tr>
<tr>
<td>Cell number of day 3 embryos</td>
<td>8.09 ± 1.27</td>
<td>7.90 ± 0.04</td>
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</table>

Values are means ± standard deviation.

Discussion

The quest for a biological marker for embryo competence that can be used for selection of embryos for transfer has spanned the history of IVF. To date, however, embryo selection based on morphological parameters such as pronuclear pattern (Tesarik and Greco, 1999; Zollner et al., 2002; Scott, 2003; Balaban et al., 2004), early cleavage (Shoukir et al., 1997), day 3 cell number and compaction (Desai et al., 2000; Fisch et al. 2001, 2003; Neuber et al., 2003), and blastocyst formation and grade (Gardner et al., 2003; Racowsky et al., 2003; Kovacic et al., 2004) have been the basis for embryo grading and selection in IVF laboratories around the world. Biochemical evaluation of embryonic metabolism has also been explored but mostly on a research basis and has failed to attain favour in clinical laboratories (Gardner et al., 2001; Roudebusch et al., 2002; Houghton et al., 2003). Preimplantation genetic diagnosis (PGD) and chromosome analysis of embryos prior to transfer has been another approach for embryo selection (Munné et al., 2003). This technique, although quite powerful, is too invasive, labour intensive and too expensive to be routinely used for all patient embryos.

The data from this preliminary trial demonstrate that embryos cultured individually in microdrops secrete detectable concentrations of sHLA-G into their culture milieu. Transfer of embryos positive for sHLA-G was associated with a higher implantation rate than transfer of sHLA-G negative embryos (P < 0.05). These data corroborate earlier findings (Noci et al., 2004; Sher et al., 2004, 2005a,b; Yie et al., 2005) suggesting that secretion of this factor may reflect embryonic potential.

Interestingly, in the trial by Noci et al. (2005) no positive pregnancy outcomes were obtained in 26 of 66 patients where all embryos transferred were negative for sHLA-G. Our experience to date has not been quite as definitive. In 25
transfers where sHLA-G-secretion was not detected in day 3 conditioned medium, it was still possible to achieve nine clinical pregnancies. Sher et al. (2005b) obtained a pregnancy rate of 25% (37/150) after the transfer of only sHLA-G negative embryos, as compared with 67% (158/237) if at least one sHLA-G embryo was transferred. One explanation might be that the timing of sHLA-G expression by embryos may differ and that a percentage of embryos not expressing sHLA-G on testing could initiate expression at a later point in development. Noci et al. (2005) showed that at 48 h post-retrieval, only 8% of embryos expressed s-HLA-G but expression increased to 48% by 72 h (day 3). In the present investigation, it was noted that a greater proportion of the more advanced cell stage embryos expressed sHLA-G (Figure 3). Yie and colleagues (2005) reported similar correlation between embryo cleavage rate and HLA-G expression. In contrast, Noci et al. (2005) could not find a correlation between overall embryo morphology/cell number and secretion of sHLA-G. Differences in laboratory protocols and culture media could potentially impact on overall timing of embryonic sHLA-G secretion. To address this question, comparative data from several different laboratories is necessary. Until its expression pattern in embryos is fully understood, sHLA-G secretion can only be taken as a secondary indicator of embryo competence after traditional grading based on morphologic parameters.

An alternate explanation for the accumulating data on sHLA-G expression and clinical outcomes might be that HLA-G secretion itself is not an absolute necessity for implantation, but rather serves only to confer some immune advantage to the embryo in escaping from NK killer cell activity (Piccinni and Romagnani, 1996; Hunt et al., 2005). The higher implantation and pregnancy rates with the transfer of HLA-G-positive embryos may simply be a reflection of their greater ability to evade the cytotoxic activity of maternal T cells as compared with embryos not secreting HLA-G. It might be interesting to assess sHLA-G secretion in day 5 culture supernatants prior to blastocyst stage transfer and relate this to cycle outcome.

Another aspect that needs consideration is the absence of pregnancy in some transfers where one or more sHLA-G-positive embryos were in fact transferred. Other mediating factors impacting implantation of sHLA-G-positive embryos may include chromosomal status. Assay of day 3 conditioned medium from PGD cases indicated that some embryos diagnosed as chromosomally abnormal still had the capacity to secrete sHLA-G (unpublished data). This and other patient-specific factors such as uterine receptivity need further investigation, along with embryo morphology and sHLA-G secretion, to gain a better perspective on the role of this molecule in the implantation process.

Performance of the sHLA-G assay was simple but data interpretation and accurate quantification were confounded by the low concentrations of sHLA-G secreted by individually cultured embryos. The low-end detection limit of the ELISA kit used in this study was 1 ng/ml, with concentrations in most culture media drops ranging from 3 to 10 ng/ml. Some media samples with very low concentrations (in the 1–2 ng/ml range) could potentially be missed with this assay system. Despite this limitation, a positive relationship between the presence of this factor and cycle outcome could still be demonstrated. However, it is clear that manufacturer control on batch variation and low end sensitivity of this kit will play a major role in whether this assay system can be applied to clinical samples for prospective screening and selection of embryos.

Assay sensitivity and reported ranges of sHLA-G secretion have varied with the different methodologies used for detection of this marker. Sher et al. (2004, 2005a,b) used a semi-qualitative in-house assay to identify the presence or absence of sHLA-G. Fuzzi et al. (2002) reported a range of 1.5–30 ng/ml in positive samples with a mean of 8.9 ng/ml in pregnant patients. Noci et al. (2005) noted a similar range and also found that four in 10 embryos expressed sHLA-G, much like the present findings. The assay described by Yie et al. (2005) had a low end detection limit of only 10 ng/ml. Embryos were group cultured (1–4 per well), so levels of secretion by individual embryos was difficult to ascertain. These investigators reported a mean of 0.165 µg/ml.

The sHLA-G results using this new ELISA kit compare favourably with what has been previously reported. Moreover, this commercial assay used a shortened protocol, allowing values to be reported within 3.5 h. The semi-qualitative assay of Sher et al. (2004, 2005a,b) also allows rapid same day reporting, but does require that the microtitre plate be precoated and incubated the day before with anti-sHLA-G antibody. These shortened same day assay systems provide a distinct advantage when used for any clinical application. Day 3 embryos could be prescreened for sHLA-G secretion prior to selection for same day transfer, thus avoiding the need for extended time in culture and transfer at the blastocyst stage.

In conclusion, a new commercial assay system for sHLA-G was tested for human IVF application. The assay offered the advantage of ease, shortened assay time and quantitative data. Resultant data corroborated other published work suggesting a relationship between implantation potential and embryonic secretion of sHLA-G. Prospective screening and addition of sHLA-G status to traditional morphological criteria for embryo selection needs to be further explored and is currently underway.

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