How to predict implantation? No correlation between embryonic aneuploidy and soluble human leukocyte antigen G-concentrations

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Objective: To determine if soluble human leukocyte antigen-G (sHLA-G) concentrations in spent culture media may assist in identifying the normal embryo for implantation.
Design: Prospective blinded comparative study.
Setting: Reproductive genetic and reproductive medicine centers.
Patient(s): One hundred and sixteen embryos obtained from eight patients undergoing in vitro fertilization (IVF) with preimplantation genetic diagnosis (PGD).
Intervention(s): Culture media obtained 2 days after fertilization were analyzed for sHLA-G concentrations using an enzyme-linked immunosorbent assay (ELISA) assay. A sHLA-G concentration of $\geq$1.9 mIU/mL was considered a positive predictor for successful implantation. Polar bodies and blastomeres from day-3 embryos were tested by PGD for 5 to 11 chromosomes: 8, 9, 13, 15, 16, 17, 18, 21, 22, X, and Y.
Main Outcome Measure(s): The results of the sHLA-G concentrations were compared with the results of the PGD analyses.
Result(s): We found an sHLA-G concentration $\geq$1.9 mIU/mL in 48% (56 out of 116) and normal PGD results in 52% (57 out of 116) of embryos. Of the embryos with normal PGD results, 46% (26 out of 57) had sHLA-G concentrations $\geq$1.9 mIU/mL. Among the embryos with sHLA-G $\geq$1.9 mIU/mL, 46% (26 out of 56) had normal PGD results, and 21% of embryos displayed both normal PGD results and sHLA-G $\geq$1.9 mIU/mL.
Conclusion(s): No correlation between concentrations of sHLA-G in embryo culture media and PGD results of an embryo's aneuploidy were observed. (Fertil Steril® 2009;91:2408–13. ©2009 by American Society for Reproductive Medicine.)

Key Words: Soluble HLA-G, preimplantation genetic screening, PGD, implantation, embryo selection, aneuploidy

Can we predict which embryo will implant and result in a pregnancy of a fetus with normal karyotype? Answering this question is becoming even more important with the current trend for single embryo transfer because of the high incidence of multiple gestations and resultant increase in preterm births and perinatal morbidity after in vitro fertilization (IVF) and embryo transfer compared with natural conceptions (1). Although single embryo transfers result in a marked decrease in multiple births, it is associated also with a decrease in pregnancy rates per cycle (2). The approach to improving pregnancy rates after single embryo transfer has focused on selection of embryos most likely to implant and end in successful live birth. Methods currently being employed to select embryos include embryo morphology (3), preimplantation genetic diagnosis (PGD) (4, 5), and measurement of soluble human leukocyte antigen-G (sHLA-G) concentrations in embryo culture media (6–9). Embryo morphology is currently the most commonly used method of selecting embryos worldwide, but its predictive value by itself is limited (10, 11). Recent publications have suggested that PGD (4, 5) and embryo culture media determination of sHLA-G concentration (6–9) have been helpful in selecting embryos that result in an enhanced live birth rate than nonselection. The question then arises as to relationship between the results of PGD and concentration of sHLA-G in embryo culture media. Our study examined whether sHLA-G can assist in identifying the normal embryo for implantation.

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MATERIALS AND METHODS

One hundred and sixteen embryos out of 133 from eight patients undergoing IVF with PGD for treatment for infertility or to conceive with a healthy baby comprised the study population. All embryos were biopsied for PGD. In four patients, PGD was performed only for aneuploidy; in other four patients, PGD was for aneuploidy and for different single-gene disorders: fragile-X syndrome, spinal muscular atrophy, hereditary nonpolyposis colorectal cancer type I, and Fanconi anemia as well as HLA typing. The polar bodies

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and blastomere biopsies for PGD were approved by the institutional review board of Reproductive Genetics Institute, and all patients signed informed consent documentation.

Preimplantation Genetic Diagnosis (PGD) Procedure

The PGD for aneuploidy was performed by fluorescence in situ hybridization (FISH) analysis of polar bodies and blastomeres, using commercially available chromosome-specific probes (Abbot, Downers Grove, IL) (5) or by polymerase chain reaction (PCR). Polar bodies and blastomeres from day-3 embryos were tested by PGD for 5 to 11 chromosomes: 8, 9, 13, 15, 16, 17, 18, 21, 22, X, and Y. The PCR was used for genetic analysis of polar bodies and blazoners for single-gene disorders or HLA typing. We performed PCR aneuploidy testing by adding primers for chromosome-specific microsatellite markers to the multiplex PCR protocols worked out for a specific genetic disorder or HLA typing. The primers for PCR-based aneuploidy testing used for identification of the copy number of chromosomes 13, 16, 18, 21, 22, X, and Y were recently described by Rechitsky et al. (12).

Determination of sHLA-G

The level of sHLA-G in embryo culture media was detected by using a specific enzyme-linked immunosorbent assay (ELISA) kit (EXBIO/BioVendor, Praha, Czech Republic). The capture antibody is a specific anti-sHLA-G monoclonal clone MEM-G9, and the detecting antibody is anti-β_2-microglobulin. The embryo culture media was added in each single-well as 50-μL droplets without oil contamination. The standard sHLA-G was diluted to give a calibration curve within the range of expected sHLA-G secretion in embryo culture media. The standard curve and extrapolation of each sample's readings were made with TableCurve2D (Systat Software, Inc, Richmond, CA) software.

Statistical Analysis

The physicians performing embryo transfer, the embryologists, and the geneticists analyzing the PGD results were blinded to the sHLA-G testing. The determination of sHLA-G was performed blindly to the results of the PGD.

The frequencies of normal and abnormal PGD results and sHLA-G results were compared using a 2×2 contingency table with Fisher's exact test (InStat; GraphPad Software, San Diego, CA). The correlation between PGD results and sHLA-G culture media concentrations was tested using linear regression. A two-tailed $P<.05$ was considered statistically significant.

RESULTS

A total of 133 embryos were generated from eight women (mean age: 32.4 years $\pm$ SD) undergoing IVF with PGD. Of these, 17 embryos were not tested for aneuploidy because they were affected by the mutation or did not develop to blastocyst stage and thus were determined unsuitable for transfer, which left 116 embryos (87%) with aneuploidy results that were included in the study. Of the 116 embryos, 57 (49%) had normal PGD results, and 59 (51%) had abnormal results. A list of the abnormal results is shown in Table 1. The sHLA-G culture media concentrations were $\geq$1.9 mIU/mL in 56 (47%) and <1.9 mIU/mL in 61 (53%).

Of the 57 embryos displaying normal PGD results, 26 (46%) had sHLA-G culture media concentrations $\geq$1.9 mIU/mL, and 31 (54%) had concentrations <1.9 mIU/mL (Fig. 1A). When considering the 56 embryos that secreted $\geq$1.9 mIU/mL sHLA-G, 26 (46%) had normal PGD results, and 30 (54%) had abnormal results (see Fig. 1B).

Linear regression revealed no correlation between PGD results and sHLA-G concentrations in the embryo culture media, with correlation coefficient $r = 0.077$ and $r^2 = 0.006$, P=.4 (Fig. 2).

The combination of normal PGD results and sHLA-G concentrations of $\geq$1.9 mIU/mL in their culture media was observed in 24 of the 116 embryos studied (21%). Two babies were born with sHLA-G of 1.9 mIU/mL, and two biochemical pregnancies also occurred with sHLA-G of 1.5 and 1.6 mIU/mL.

DISCUSSION

Our data show no correlation between PGD results of an embryo's aneuploidy and concentrations of sHLA-G in the embryo culture media. This finding suggests that chromosomal

TABLE 1

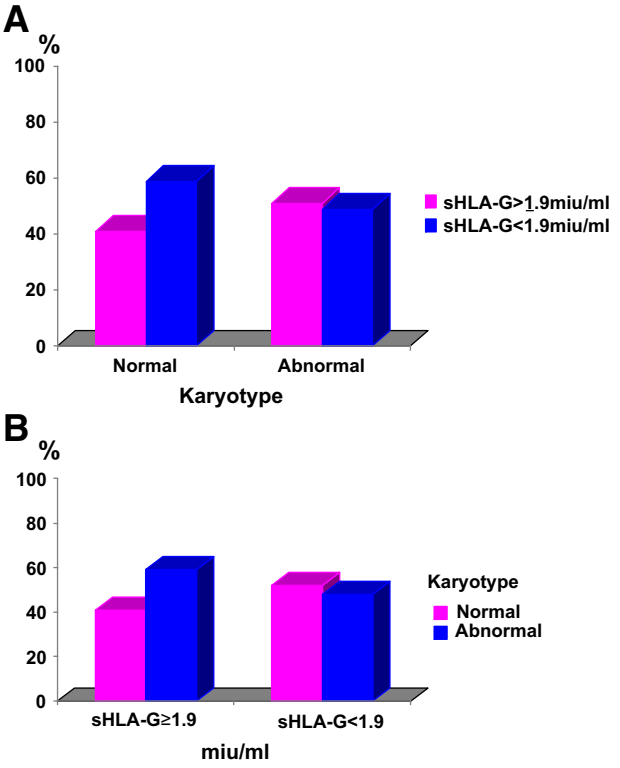
Chromosomal abnormalities found in 116 embryos undergoing preimplantation genetic screening.

Chromosomal abnormality	N
+9	1
−13	1
+13–22	1
−16	2
+16	4
−18	1
+18	5
−21	1
+21	11
−22	4
+22	4
−21–22	2
+21+22	2
Chaotic	9
XO	1
Polyploid	4
Haploid	1

Coulam. No correlation of aneuploidy and sHLA-G. Fertil Steril 2009.

FIGURE 1

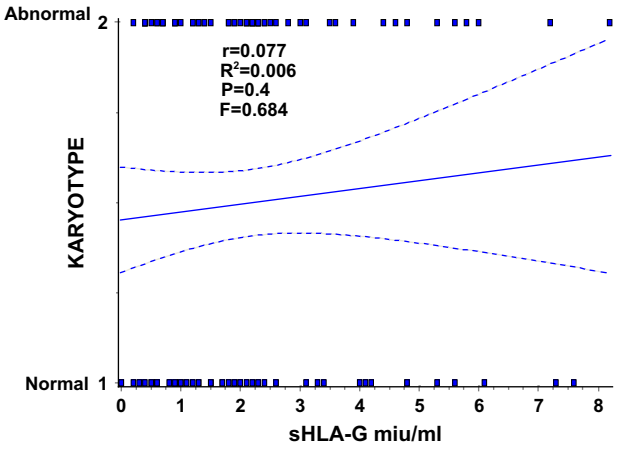
Percentage of PGD results and sHLA-G culture media concentrations. (**A**) Of the 56 embryos that secreted ≥1.9 mIU/mL sHLA-G, 23 (41%) had normal PGD results, and 33 (59%) had abnormal results. (**B**) Of the 57 embryos displaying normal PGD results, 26 (46%) had sHLA-G culture media concentrations ≥1.9 mIU/mL, and 31 (54%) had concentrations <1.9 mIU/mL.



Coulam. No correlation of aneuploidy and sHLA-G. Fertil Steril 2009.

FIGURE 2

Linear regression analysis showing no correlation between PGD results and sHLA-G concentrations in embryo culture media with correlation coefficient $r = 0.077$ and $r^2 = 0.006$, $P = .4$.



Coulam. No correlation of aneuploidy and sHLA-G. Fertil Steril 2009.

abnormality and decreased secretion of sHLA-G by embryos are independent markers of risk for lack of implantation. Soluble HLA-G concentrations in spent culture media cannot identify a normal embryo for implantation without aneuploidy testing by PGD. Because abnormal embryos do implant, it is not surprising that no correlation was found. One possibility that needs further investigation is to identify level of sHLA-G that may predict which of the normal embryos available for transfer would implant.

Although both PGD results (4, 5) and diminished concentrations of sHLA-G in embryo culture media (6–9) have been shown to be risk factors for unsuccessful implantation, each by itself has limitations. By selecting only chromosomally normal embryos for transfer, PGD was expected to improve IVF outcome. To date, over 20,000 PGD procedures have been performed worldwide (13) and controversy still exists regarding its efficacy (13–15). Indeed, one recently published large randomized study indicated that PGD was detrimental to pregnancy outcome (16). Three explanations have been put forward to address this controversy. The first explanation involves the number of chromosomes studied when performing PGD. The number of aneuploidic embryos demonstrated increases with the number of chromosomes investigated. Technical limitations of using FISH restrict the number of chromosomes that can be screened. Most laboratories offering PGD services currently examine 5 to 12 chromosomes per embryo. Screening for aneuploidies within the chromosomes most often found to be abnormal in prenatal samples has been successful in reducing the number of such aneuploidies (17), but a significant improvement in embryo implantation could not be shown (18). This observation suggests that aneuploidy for additional untested chromosomes is occurring, compromising the embryo implantation rate. It is hoped that newer techniques using microarray comparative genomic hybridization (CGH) (19) or gene chips (20) will help to address the issue of the number of chromosomes investigated for PGD.

A second explanation for the discrepant results describing pregnancy and live birth rates after PGD has been a substandard application of PGD techniques (21). Munne et al. (21) stated that, without expert biopsy sampling leading to minimal embryo damage and expert fixation methods leading to low error rates, the benefits of PGD cannot be realized. A third explanation for the lack of improvement of pregnancy and live birth rates after PGD involves the major pitfall of aneuploidy screening with a single blastomere biopsy and the existence of mosaicism (22–24). Chromosomally normal and abnormal blastomeres can coexist within the same early-cleavage-stage embryo (22). A recent report described the rate of mosaicism among day-3 embryos to be 50% (23), and a discordance rate of over 70% was noted when two blastomeres were biopsied from the same embryo on day 3 and

analyzed for eight chromosomes (25). Because of these findings, the Practice Committee of the American Society for Reproductive Medicine has recently reported an opinion that "available evidence does not support the use of Preimplantation Genetic Screening as currently performed to improve live birth rates in patients with advanced maternal age, previous implantation failure or a history of recurrent pregnancy loss" (26). Because in our study the polar bodies were also tested, the possible mosaicism is very low.

HLA-G is a nonclassic major histocompatability (MHC) class I antigen (27) that has been proposed to have a pivotal role in the protection of the allogenic embryo from the maternal immune system (28). In 1996, Jurisicova first reported HLA-G heavy chain mRNA was present in 40% of blastocysts, in some cleavage stage embryos, and in unfertilized oocytes (29). In concordance with mRNA data, a similar proportion of embryos stain positive for HLA-G immunohistochemistry (30). Alternative splicing of its primary transcript gives rise to both membrane-bound and soluble isoforms of HLA-G (31). Although interaction between soluble forms of HLA G (sHLA G) secreted by the trophoblasts and uterine lymphocytes in the decidua has been proposed to be a major player in the induction of immunotolerance for the invading blastocyst (28), the natural killer (NK) cell receptors responsible for HLA-G recognition have remained controversial. Initial studies suggested that killer-cell immunoglobulin-like receptor (KIR) molecules on NK cells interact with HLA-G (32). However, these findings have not been confirmed by other independent laboratories (33, 34). Until recently, it seemed that NK cell recognition of HLA-G by peripheral blood or decidual NK cells could be explained by the CD94/NKG2A receptor interacting with HLA-E containing and HLA-G–derived leader peptide in the antigen-binding groove or, in a minority of cases, by direct HLA-G recognition by immunoglobulin-like transcript 2 (ILT2) on an NK cell subset (33, 34). Ponte et al. (35) and Rajagopalan and Long (36) have more recently implicated a receptor encoded by the KIR family in HLA-G recognition, designated KIR2DL4. Rajagopalan and Long (36) have shown by genetic transfer that the KIR2DL4 molecule inhibits NK cell lysis of target cells expressing HLA-G. Both Rajagopalan and Long (36) and Cantoni et al. (37) have demonstrated direct binding of KIR2DL4 fusion proteins to HLA-G, confirming the specificity of this interaction. Furthermore, Ponte et al. (35) reported that KIR2DL4 is expressed on a substantial proportion of NK cells in the decidua during the first trimester of pregnancy. Soluble HLA-G suppresses NK cytotoxicity (38), stimulates interferon-γ and tumor necrosis factor α production by NK cells (39), and induces apoptosis of $CD8^+$ T cells (40) as well as stimulates production of transforming growth factor β-1 (TGF-β1) by activated macrophages (41). Taken together, these observations suggest that the function of HLA-G is to modulate cytokine secretion to induce immunotolerance; however, nonimmune functions to allow for successful embryo implantation and pregnancy maintenance, including controlling trophoblast invasion and contributing to vascular remodeling of spiral arteries, have also been proposed (42). Because the gene for HLA-G is located on chromosome 6 and aneuploidy for chromosome 6 only occurs in 6% (12), this does not explain our results.

Although determination of sHLA-G concentrations in embryo culture media (6–9) has been shown to be helpful in selecting embryos that result in an enhanced implantation rate compared with nonselection (6–9), it, too, has its limitations. Similar to the findings in our study, a previous report found that half of the embryo culture media tested had sHLA-G concentrations $\geq$1.9 IU/mL and half had $<$1.9 IU/mL (9). However, only half of the embryos with HLA-G concentrations $\geq$1.9 mIU/mL implanted after they were transferred into the uterus, giving a positive predictive value for implantation of 50% (9). That not all embryos whose culture media had sHLA-G concentrations $\geq$1.9 mIU/mL resulted in pregnancy suggests that sHLA-G is essential for but not sufficient for implantation to occur.

In other words, not all embryos fail to implant for the same reason. The goal would be to identify multiple risk factors for implantation failure and to test for multiple analytes. By identifying the embryo most likely to implant and lead to a live birth, the ultimate goal of single embryo transfer and singleton birth for each cycle would be enhanced. Achieving this goal would answer a number of questions regarding the medical and economic concerns currently plaguing assisted reproductive procedures.

Human reproduction is an inefficient process, with only 22.8% of conceptive matings resulting in live birth (43). Failure of implantation accounts for 75% of conceptions that are lost (44) and is the most common cause of lack of successful pregnancy after IVF and embryo transfer. Among infertile women, implantation rates of approximately 15% per embryo transferred after IVF render the process of assisted reproduction inefficient (45). To try to adjust for the inefficiency, multiple embryos are transferred, increasing the risk of multiple gestations. Multiple gestations are associated with increased maternal mortality (46) and perinatal morbidity and mortality (47). The risks of preterm birth and perinatal mortality are increased after IVF as a result of increased multiple gestations (1, 48). The only way to decrease these risks is to transfer single embryos. A number of studies have shown the success of decreasing the multiple gestation rate with (2, 49) and without (50–52) decreasing the pregnancy rate per cycle in both younger ($<$36 years) (2, 49–52) and older (36 to 39 years) (53) women, especially if frozen embryo transfers are considered in the calculation (49, 51, 54). Single blastocyst stage transfers resulted in a statistically significantly higher delivery rate than single cleavage stage embryo transfers (32% vs. 22%) (55). Although all studies demonstrated a decrease in multiple gestations with single embryo transfers, concern has been expressed that the twinning rate is reduced at the expense of declining pregnancy rates, a need for more treatment cycles, a potential delay in treatment success, and higher treatment costs (56). However, economic studies have shown that transfer of a single top quality embryo can be less expensive than double embryo transfers (46, 57, 58). It is, therefore,

important to be able to select the top quality or single embryo most likely to succeed.

Of interest was the observation that only 21% of embryos tested displayed both normal PGD results and sHLA-G concentrations $\geq$1.9 mIU/mL in their respective culture media. Epidemiologic data suggest that 20% of women conceive each cycle, even when couples are fertile (59). Because abnormal embryos do implant, prospective clinical trials are necessary to answer the question of the effectiveness of embryo selection using both PGD for aneuploidy and sHLA-G.

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