Soluble MHC Class I chain-related molecule serum levels are predictive markers of implantation failure and successful term pregnancies following IVF

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BACKGROUND: Despite ongoing progresses of IVF techniques, biomarkers predicting their outcome prior to IVF initiation are lacking. We investigated whether serum levels of the stress-inducible soluble major histocompatibility complex Class I chain-related molecule, MICA, (sMIC), a regulator of cellular immunity, can be predictive of implantation or pregnancy failure after IVF. METHODS: sMIC serum levels, evaluated during the follicular phase of the cycle preceding in vitro fertilization, in a cohort of 170 infertile women with 22.3% IVF success rate were analyzed in association with implantation/pregnancy failure or live birth outcomes after IVF. RESULTS: sMIC serum levels, detected in 38% of all women undergoing IVF, were shown to be predictive both of implantation failure (≥2.45 ng/ml cut off, odds ratio (OR) = 4.6; 95% confidence interval (CI) = 1.08–19.79; P = 0.031) and successful pregnancy (<2.45 ng/ml, OR = 13.8; 95% CI = 2.03–118.3; P = 0.002). When successful implantation occurred, sMIC levels >3.2 ng/ml were predictive of spontaneous abortion (OR = 35; 95% CI = 1.74–703; P = 0.026). CONCLUSIONS: sMIC is thus to be considered as a novel blood biomarker which, when quantified prior to initiation of IVF, anticipates chances for infertile women to give birth to a viable baby. Considering medical and psychological cost of IVF, this non-invasive assay may thus contribute to better counseling, treatment and care of infertile couples prior to IVF.

Keywords: natural killer; major histocompatibility complex Class I chain-related; IVF; implantation failure; assisted pregnancy

Introduction

Diagnosis and management of infertility are a prevalent health concern in young adults (Smith et al., 2003). Ongoing progresses of IVF techniques are challenging reproductive alternatives for infertile couples. IVF is nevertheless associated with increased risks of clinical complications that represent drawbacks to their indication in women, including risks of multiple births, ectopic pregnancy, spontaneous abortion and pre-term delivery. Considering these clinical risks and the high economic cost of these advanced technique, IVF treatment is still difficult to access for most infertile couples. Although better comprehension of mechanisms that influence fertilization and implantation has provided a more accurate view of parameters associated with IVF success, the search for biomarkers that may predict pregnancy issues after IVF, which can be assessed before initiation of treatment, remains a major challenge. Most of the markers that predict embryo implantation or spontaneous abortion (Tong et al., 2004), such as embryo and endometrial qualities, occur after IVF treatment, thus limiting their impact for counseling and anticipation of stress and clinical problems associated with pregnancy failure. Prediction of the chances for infertile women to give birth to a viable baby after IVF treatment thus remains a major issue in the optimization of the medical response to increasing demands of infertile couples. The aim of our study was thus to search for markers that may improve counseling and treatment of infertile couples, as predictors of spontaneous abortion and implantation failure rates associated with IVF at a stage that precedes decision to initiate IVF treatment and expose women to potential clinical complications.

The immunological paradox of pregnancy that leads to implantation, acceptance and development of the semi
Uterine natural killer (NK) cells (uNK) are the predominant lymphoid cell population found at the embryo implantation site and progressively disappear after mid-gestation (Moffett-King, 2002). Recent insights have led to the view that uNK cell/trophoblast interactions are not harmful for the fetus but rather beneficial to establish placental vascularisation and its subsequent development. Although NK cell potential functions at the maternal-fetal interface are not yet clearly established, uNK cells are thought to participate in control of uterine vascular remodeling (Pijnenborg et al., 2006), extravillous trophoblast invasion (Hanna et al., 2006) and local anti-viral activity. In particular, uNK receptors recognize classical human leukocyte antigen (HLA)-C and non-classical major histocompatibility complex (MHC) (HLA-E, HLA-G) trophoblast ligands, that may prevent fetal attack by the maternal immune system and also regulate uNK cell cytokine/chemokine production. Presence in embryo supernatants of soluble non classical MHC molecule HLA-G, a known ligand of NK cell receptor, which expression at the feto-maternal interface modulates NK cell mediated cytokine production (Rajagopalan et al., 2006; van der Meer et al., 2006), has been correlated to higher embryo implantation rates after IVF (Puzzi et al., 2002; Warner et al., 2004). Interactions between the uNK maternal killer immunoglobulin receptors repertoire and fetal HLA-C are also thought to influence reproductive success (Hiby et al., 2004; Parham, 2004; Wu et al., 2004). In addition, uNK have unique phenotypic and functional features that differ from their peripheral blood NK cell counterparts (Tabiasco et al., 2006). NK cells secrete angiogenic factors and cytokines that favor implantation and placentaion (Ashkar et al., 2003; Coulam et al., 2003; Hanna et al., 2006; Ledee-Bataille et al., 2004; Moffett-King, 2002). Any dysfunction of uNK cells may thus represent a drawback to successful implantation and pregnancy.

Although functional implications of the expression of stress-inducible MHC class I related (sMIC) molecules are widely explored in the field of oncology (Carbone et al., 2005; Groh et al., 2005; Groh et al., 1998) and auto-immunity (Hue et al., 2004; Meresse et al., 2004), it has, to our knowledge, never been investigated with regards to reproduction failure. Stress induced NKG2D ligands expression on allo-genic or autologous cells has been shown to target NK cell cytotoxicity and cytokine/chemokine production (Andre et al., 2004; Groh et al., 2003; Lanier, 2005; Meresse et al., 2004; Raul et, 2003; Sutherland et al., 2002), both mechanisms potentially important for embryo implantation and pregnancy outcome. Dual stimulatory and inhibitory mechanisms have been associated with engagement of soluble MIC by the NKG2D receptor. Indeed, the release of a soluble form of sMIC in the serum of some cancer patients has shown to induce internalization of the stimulatory NKG2D receptor in effector NK and T lymphocytes, thus impairing both innate and adaptive anti-tumor immune responses and an escape mechanism favoring tumor growth (Coudert et al., 2005; Groh et al., 2002; Hayakawa et al., 2006; Jamieson et al., 2002; Raul et, 2003; Smyth et al., 2005; Wu et al., 2004). Such down regulation of NKG2D by placental-derived sMIC has also recently been suggested as an immune escape mechanism that may down regulate maternal immune responses during pregnancy (Mincheva-Nilsson et al., 2006).

We here investigated whether the stress inducible sMIC could be detected in serum of women that fail to become pregnant or to give birth to a viable baby after IVF.

Subjects and Methods

This prospective study, approved by the local ethics committee, was performed on a consecutive series of infertile patients who underwent an IVF, with or without ICSI, at the Center of Assisted Reproductive Medicine, La Conception hospital in Marseilles (January 2004–October 2005). A cohort of 170 infertile women, all candidates for IVF, was recruited for this study after given consent. Clinical indications were unexplained infertility, male infertility and tubal factor. None of the women included had a history of previous pregnancy. Plasma samples were collected during the follicular phase of the cycle preceding IVF. All patients received a similar stimulation regimen. Ovarian stimulation was performed by using recombinant FSH (Gonal F®, Serono Pharma, Paris, France; Puregon®, Organon France, Paris, France) started after pituitary down-regulation with GnRH agonist analog (LHRH analog antagonist). Complete pituitary desensitization was confirmed by both low plasma estradiol (E2) below 50 pg/ml and ultrasound examination to exclude ovarian cyst and confirmed the endometrial thickness below than 5 mm. 10 000 IU HCG was administered when at least three follicles exceeded 16 mm in diameter. Oocyte recovery was performed by transvaginal ultrasound guidance and general anesthesia 32–34 h after HCG administration. Luteal phase was supported with natural progesterone from the day of embryo transfer. The embryo transfer was performed on the second or third day post-oocyte collection. A single serum HCG measurement was performed 15 days after embryo transfer. A clinical pregnancy was defined when an intra uterine gestational sac with fetal heartbeat was detected by transvaginal ultrasonography.

Collected oocytes were cultured in a four-well multi-dish with 600 μL of culture medium added with serum substitute supplement. Each well contained from one to four oocytes. IVF or ICSI technique was used for insemination. Oocytes fertilization was observed 16–18 h after insemination under an inverted microscope and fertilization rate was calculated. Embryos were examined after 48 h or 72 h in culture to assay the rate of cleavage and choose up to four embryos for transfer. The embryos were graded according to the number of blastomers and the amount of fragmentation. The grades used were: grade 1 (no fragments), grade 2 (<20% fragmentation), grade 3 (20–50% fragmentation), grade 4 (>50% fragmentation). Twenty-five non pregnant women volunteer blood donors between 20 and 38 years, with no infertility background, no spontaneous abortion or fetal complications history, that experienced past normal pregnancies giving birth at term to at least two healthy babies were recruited as controls.

Enzyme-linked immunosorbent assay of sMIC levels in plasma

sMIC concentrations were evaluated in the plasma using a sandwich enzyme-linked immunosorbent assay as described (Hue et al., 2004). The detection threshold of recombinant soluble MICA protein was 0.1 ng/ml.

Statistical analysis

Differences between groups were evaluated for statistical significance by Student’s t test or Mann-Whitney rank sum test, depending on whether the data were normally distributed, using the GraphPad Prism software version 4.0b. Chi-Square analysis was used to
compare sMIC frequencies between various independent groups. Receiveroperating characteristic (ROC) curve analysis was used to analyse sMIC concentration cut off points and their sensitivity/specificity. The cut off determined was further used to evaluate odds ratio (OR). Significant P values were set as <0.05.

**Results**

**Higher sMIC serum levels are found in infertile women that experience implantation failure after IVF**

Of the 170 patients included, 38 experienced successful embryo implantation following IVF, among which 30 ongoing pregnancies gave birth to a viable baby at term and eight spontaneous abortions occurred after successful implantation. None of the patients had previous history of pregnancy or pregnancy losses. Three patients experienced spontaneous abortion at 8SA, two patients at 9SA, two patients at 6SA, and one patient at 7SA. Pathology reports (realized for >8SA losses) were not in favor of genetic losses. Patient’s characteristics are summarized in Table 1.

Sixty-four (38%) patients undergoing IVF had detectable sMIC serum levels (median = 9.35 ng/ml, 25–75 percentile: 2.45–19.95). A similar frequency of 32% women with detectable sMIC was evaluated in a control group of non pregnant fertile women, that had successful pregnancy history of at least two born children (median = 3.65 ng/ml, 25–75 percentile: 1.75–4.40).

Despite the fact that parameters associated to IVF outcome where normalized in these groups (Table 1), median sMIC levels in sMIC-positive patients significantly differed in women that had successful embryo implantation (median = 2.50 ng/ml, 25–75 percentile: 1.50–9.70) as compared to the implantation failure group (12.00 ng/ml, 3.00–24.25, P < 0.021). We thus further investigated whether sMIC levels may help prediction of implantation failure in sMIC positive women. We thus defined a 2.45 ng/ml cut-off value allowing risk evaluation by ROC analysis (sensitivity 82, specificity 50%). The 75% sensitivity refers to the same cut off defined below is used to evaluate different groups. sMIC levels ≥2.45 ng/ml were associated with implantation failure (OR: 4.6, 95% confidence interval (CI) 1.08–19.79, P = 0.031). sMIC serum levels >27.70 ng/ml were always associated with lack of embryo implantation after IVF (Fig. 1).

**sMIC serum levels predicts chances to have a viable born baby at term after IVF**

In addition to predicting implantation failure, we further prospectively assessed whether increased sMIC levels could predict IVF outcome as birth of a live baby. Indeed after implantation was successful (38 women), eight women experienced a spontaneous abortion after IVF and 30 gave birth to a viable baby. When positive, soluble serum MIC values observed before IVF were found to be significantly lower in the group of women that gave birth after IVF (median = 2.10 ng/ml, 25–75 percentile: 1.50–2.50) in reference to women who experienced spontaneous abortion or implantation failure after IVF (median = 11.12 ng/ml, 25–75 percentile: 3.25–22.50, Fig. 1). We thus further evaluated the value of sMIC that may predict chances of achieving successful pregnancy instead of spontaneous abortion or implantation failure. Using the same cut-off of 2.45 ng/ml (sensitivity = 82% and specificity = 75%), the chances to have a viable baby were higher when predicted by sMIC serum levels prior to IVF lower than the 2.45 ng/ml threshold (OR = 13.8, CI 95% = 2.03–118, P = 0.002). Highest values of sMIC observed in women achieving ongoing pregnancies were 6 ng/ml (Fig. 1).

**High values of sMIC are associated to spontaneous abortion after implantation success**

Although the frequency of sMIC positive women did not significantly differ in relation to implantation success (36% in the implantation success versus 38% in implantation failure group), when implantation was successful (38 cases), frequencies of sMIC positive women were higher in the group of women experiencing spontaneous abortion (75%) than in that with successful term pregnancies (26%, P = 0.03).

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**Table 1: Characteristics of the 170 infertile women recruited in the study**

<table>
<thead>
<tr>
<th></th>
<th>Non-pregnant implantation failure</th>
<th>Spontaneous abortion after implantation</th>
<th>Evolutive pregnancy live born baby</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>132</td>
<td>8</td>
<td>30</td>
<td>NA</td>
</tr>
<tr>
<td>Age (years) (mean ± SD)</td>
<td>32.07 ± 3.742</td>
<td>32.00 ± 3.721</td>
<td>32.51 ± 3.373</td>
<td>0.88</td>
</tr>
<tr>
<td>Tubal factors (%)</td>
<td>29.25</td>
<td>–</td>
<td>24.6</td>
<td>ns</td>
</tr>
<tr>
<td>Unexplained (%)</td>
<td>41.25</td>
<td>66</td>
<td>44</td>
<td>ns</td>
</tr>
<tr>
<td>Duration of infertility (years) (mean ± SD)</td>
<td>4.38 ± 2.221</td>
<td>4.37 ± 2.12</td>
<td>4.64 ± 3.58</td>
<td>0.67</td>
</tr>
<tr>
<td>Basal FSH levels (UI/l) (mean ± SD)</td>
<td>7.15 ± 2.19</td>
<td>7.23 ± 1.86</td>
<td>6.99 ± 2.85</td>
<td>0.93</td>
</tr>
<tr>
<td>Basal LH levels (UI/l) (mean ± SD)</td>
<td>4.94 ± 1.37</td>
<td>4.77 ± 1.10</td>
<td>4.45 ± 1.46</td>
<td>0.84</td>
</tr>
<tr>
<td>Gonadotrophin (UI)</td>
<td>2120 ± 962.9</td>
<td>2125 ± 831.6</td>
<td>2299 ± 678.2</td>
<td>0.63</td>
</tr>
<tr>
<td>Endometrium (mm)</td>
<td>10.60 ± 1.91</td>
<td>11.40 ± 0.89</td>
<td>10.86 ± 1.86</td>
<td>0.69</td>
</tr>
<tr>
<td>Duration of stimulation (days)</td>
<td>12.12 ± 1.68</td>
<td>10.42 ± 1.92</td>
<td>11.74 ± 2.37</td>
<td>0.52</td>
</tr>
<tr>
<td>E2 (UI) on HCG day</td>
<td>2001 ± 939.5</td>
<td>2268 ± 1391</td>
<td>1912 ± 1018.4</td>
<td>0.65</td>
</tr>
<tr>
<td>Number of oocytes collected (mean ± SD)</td>
<td>9.5 ± 5.41</td>
<td>8.25 ± 3.19</td>
<td>10.2 ± 4.49</td>
<td>0.61</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>77.25 ± 16.92</td>
<td>72.56 ± 31.62</td>
<td>74.13 ± 16.76</td>
<td>ns</td>
</tr>
<tr>
<td>Number of embryos available for transfer (mean ± SD)</td>
<td>6.77 ± 3.74</td>
<td>6.12 ± 3.98</td>
<td>7.13 ± 3.53</td>
<td>0.75</td>
</tr>
<tr>
<td>Mean of embryos transferred</td>
<td>2.044 ± 0.41</td>
<td>1.8 ± 0.42</td>
<td>2.0 ± 0.0</td>
<td>0.25</td>
</tr>
</tbody>
</table>
Further more, when positive, median sMIC serum levels prior IVF were shown to be higher in the group that experienced spontaneous abortion after IVF (median $= 9.70$ ng/ml, 25–75% percentile: 2.47–23.90) than in the group successfully achieving pregnancy (median $= 2.10$ ng/ml, 25–75% percentile: 1.50–2.50). We thus further evaluated whether the risk of spontaneous abortion could be predicted from sMIC serum levels. We could show that, when implantation is successful after IVF, higher sMIC were associated with miscarriage occurring during pregnancy obtained after IVF. In contrast, higher sMIC serum levels did not appear to associate to occurrence of miscarriage in pregnant women that did not undergo IVF (data not shown). Indeed, using a cut-off value of sMIC serum levels $> 3.2$ ng/ml (sensitivity 83% and specificity 75%), sMIC could predict spontaneous abortion after successful implantation when compared to term pregnancy (OR = 35, $P = 0.026$, 95% CI 1.74–703). No birth at term was observed when women had sMIC serum levels $> 6$ ng/ml.

**Discussion**

NK cells play an essential role at early stages of implantation, as they represent the predominant immune partners controlling cytokine/chemokine/angiogenic factor production and trophoblast modeling (Sargent et al., 2006). sMIC is one of the numerous ligands for the activating NKG2D receptor, widely expressed on NK and CD8 and $\gamma\delta$ T lymphocytes. The mechanisms by which stress inducible soluble MHC Class I chain-related molecules may influence embryo implantation remains unclear. The engagement of activating NKG2D receptors by NKG2D ligands, including sMIC, has been demonstrated to deliver both activating and inhibitory signals to the immune system. Soluble NKG2D ligands are indeed co-stimulatory signals for NK-mediated cytotoxic activity, proliferation and cytokine production (Andre et al., 2004; Bryceson et al., 2006; Raulet, 2003; Sutherland et al., 2002; Upshaw et al., 2006).

Alternatively, sMIC-induced internalization of its NKG2D receptor has been described as a mechanism down regulating anti tumoral NK cell activity (Wu et al., 2004). In this regard, down regulation of NKG2D by placental-derived sMIC has recently been suggested as an immune escape mechanism favoring fetal survival (Mincheva-Nilsson et al., 2006) and rather indicate MIC as being associated with an immunotolerance state during pregnancy. Still, in multiparous fertile women, tested at distance from pregnancies we failed to detect sMIC levels, suggesting that in contrast to infertile women or
normal pregnancies (Mincheva-Nilsson et al., 2006), high sMIC levels are not a common feature of non pregnant fertile women.

Our main finding is thus that the stress inducible immunostimulatory MHC class I Chain-Related molecule, is prevalent before IVF in women that will experience implantation and pregnancy failure. Serum levels of sMIC greater than 2.45 ng/ml are predictive of higher implantation failure rates and sMIC serum levels >6 ng/ml never resulted in a term pregnancy, while levels >28 ng/ml were always associated with IVF failure. Furthermore, after implantation is successful, women that bear sMIC levels >3.2 are at high risk of experiencing spontaneous abortion after IVF. Still, mechanisms that relate sMIC to implantation and pregnancy failure, in particular its origin and impact on decidual NK cell function, remain to be further elucidated. The infertility status -related mechanisms that may contribute to enhanced sMIC protein levels in the serum of women that will not achieve successful pregnancy after IVF remain to be unraveled. The release of sMIC from the membrane of MIC-expressing cells has been reported to involve cleavage by metalloproteinase (Waldhauer et al., 2006). Altered metalloproteinase activity has been reported in IVF patients with recurrent implantation failure (Shibahara et al., 2005). Further investigation of the mechanisms generating sMIC and the impact of sMIC on immune functions associated with implantation failure or pregnancy loss after IVF are now challenging issues to explore. The fact that sMIC is also a marker of auto and alloimmune processes, as contributed by co-authors of this study in celiac disease (Hue et al., 2004; Meresse et al., 2004), where higher prevalence of infertility is reported (Meloni et al., 1999), suggests sMIC may be the signature of a higher auto or alloreactive potential of the mother to reject the embryo.

Altogether, considering high economic and psychological implications of IVF, this study is the first to provide arguments that serum sMIC quantification may be considered as a novel parameter with applications in the non-invasive evaluation and prediction of IVF outcome. Besides its prognostic value, a major advantage of this biomarker is its dosage prior initiation of women hormonal conditioning treatment. These features, if confirmed by other study cases, should thus improve counseling or management of IVF associated-risks and thus provide consequent benefits for infertile women health care.

Although predictive value of sMIC serum levels for implantation failure needs further evaluation and follow up in larger cohorts of infertile women before its application in clinical practice, its identification as a marker of IVF outcome opens a challenging field of investigation with potential implication for NK cell biology.

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