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New pre-conception immune biomarkers for clinical practice: interleukin-18, interleukin-15 and TWEAK on the endometrial side, G-CSF on the follicular side

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ABSTRACT

Identification of biomarkers of optimal uterine receptivity to the implanting embryo as well as biomarkers of oocyte competence would undoubtedly improve the efficiency of assisted reproductive technology (ART). Expression of IL-15 and IL-18 has been shown to be different in patients with failed implantation after IVF/ICSI compared with fertile controls and both correlate with local uNK (CD56+) recruitment and angiogenesis. Tumor necrosis factor weak inducer of apoptosis (TWEAK) has been described in mice as a potent early immune regulator able to protect the conceptus. The results of our studies in human suggest that TWEAK modulates the IL-18 related cytotoxicity of uNK cells. Quantification of IL-18, TWEAK and IL-15 mRNA expression by real-time PCR in endometrial tissue collected in mid-luteal phase of non-conception cycles allowed documentation of physiological events that occur at the time of uterine receptivity. Such information may be useful for the physician especially in patients where embryos fail to implant. Cytokine quantification may assist in understanding the mechanisms leading to repeated IVF/ICSI failure: either depletion of cytokines necessary for the apposition-adhesion, or an excess of cytokines leading to local cytotoxicity, may impair the implantation of the embryo. Other new data suggest that a pre-conception dialogue mediated by the oocyte and the follicular fluid and the oocyte may contribute to later implantation success. Follicular concentration of G-CSF appears as a useful biomarker of oocyte competence before fertilization. Moreover both in human and animal models, evidence of a role of the endometrium as a biosensor of the embryo is emerging.

1. Introduction

In the clinic, less than 5% of oocytes collected and only 20–25% of embryos transferred lead to a birth (Patrizio and Sakkas, 2009). Optimisation of embryo competence as well as corresponding uterine receptivity is an absolute requirement to improve success in reproductive medicine. Such objectives rely on a better understanding of the pre- and peri-conception dialogue. The success of implantation depends on a receptive endometrium, a normal blastocyst and synchronized cross-talk at the maternal–fetal interface. A cascade of cytokines, chemokines and growth factors mediate this dialogue before fertilization of the oocyte and before implantation in the endometrium (Guzeloglu-Kayisli et al., 2009; Red-Horse et al., 2004;

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Salamonsen et al., 2007; van Mourik et al., 2009). To achieve the fertilization step, a good quality oocyte must meet a normal sperm with low DNA damage, leading to the development of a functionally normal blastocyst able to communicate with the maternal endometrium. The progression of pregnancy then requires immunological tolerance which allows conceptus survival. Such cross-talk involving both the immune and endocrine systems is crucial to prevent implantation failure. A better knowledge of the uterine–oocyte/embryo interaction and regulation of both the "seed and soil" during endometrial implantation is mandatory to increase the efficacy of assisted reproduction technology. Adequate coordination between embryo and mother is indeed crucial. In this short review, we will focus exclusively on some specific cytokines – interleukin-18 (IL-18), IL-15 and tumor necrosis factor weak inducer of apoptosis (TWEAK) in the endometrium and granulocyte colony-stimulating factor (G-CSF, CSF3) in the follicular fluid – since they appear as potential biomarkers to document the local pre-conception environment and its equilibrium. Such immune biomarkers may be clinically useful for understanding mechanisms of implantation failure after embryo transfer.

2. Endometrial uterine receptivity: vascular remodeling and the underlying abnormal cytokine network

It has been proposed that uterine natural killer (uNK) cells can exert, directly or indirectly, both positive and negative control over the early events of implantation (Leonard et al., 2006; van den Heuvel et al., 2005). These cells secrete an array of cytokines important for adequate local immune regulation, angiogenesis, placental development, and establishment of pregnancy (Zhang et al., 2010). Implantation failure, recurrent miscarriage and preeclampsia have several recognized causes in common, but in most cases the precise etiology remains obscure. Various studies have identified the importance of the local immune environment and indicate the need to develop clinical tools to explore these endometrial dysregulations (Kwak-Kim and Gilman-Sachs, 2008; Quenby and Farquharson, 2006; Tuckerman et al., 2010).

Indeed the semantic distinction between implantation failure, abortion and preeclampsia might in fact be more quantitative than qualitative (Chaouat, 2008). Dysregulation of events occurring immediately after or even before conception is important determinants of embryo fate and pregnancy success. An important subset of implantation defects/early abortion cases is the consequence of a deregulation of the interleukin, tumor necrosis factor (TNFα) and interferon systems, as well as networks controlled and mediated by uNK cells. Both deficient and excess expression of cytokines, as well as immune cell numbers and activation phenotype, play key roles in implantation, since these actors can have both positive and negative effects.

In our laboratory, we observed in human and mice that a proper balance in the IL-12, IL-18 and IL-15 and a correct NK activation state result in implantation and successful pregnancy. Conversely, imbalances in these parameters correlate with implantation failure or early pregnancy loss (Ledee-Bataille et al., 2005). Their expression controls the local recruitment of CD56+ uNK cells and associated sub-endometrial angiogenesis, as reflected by the endometrial vascular flow index determined by three-dimensional ultrasound (Ledee et al., 2008a,b). Such expression patterns are reproducible from one cycle to another for the same patient, and are strongly down-regulated by high concentrations of oestrogen (Ledee et al., 2006). The excellent correlation between IL-15 mRNA expression and the sub-endometrial vascular flow index suggests that this cytokine and the uNK cells that produce it participate in the local control of angiogenesis (Ledee et al., 2008a). Abnormal sub-endometrial vascularisation assessed by ultrasound may be the consequence of distinct cytokine dysregulation patterns. These may cause implantation failure through abnormal (insufficient or excessive) recruitment of uNK cells or through inadequate endothelial vascular remodeling before implantation.

3-D ultrasonography with vocal analysis may inform on uterine preparation for a constructive dialogue with the conceptus, and ability to provide adequate embryotrophic factors and undergo angiogenesis. However in the clinic, most ultrasonic assessments are performed either at the beginning of the cycle to evaluate the ovarian reserve or in late proliferative phase just before triggering ovulation. Much more rarely, ultrasonic assessments are performed in the mid luteal phase which is clearly the only phase relevant for this issue. Focusing on vascularisation at the time of uterine receptivity to embryo implantation is absolutely mandatory from a physiological point of view (Ledee, 2005). Patients with low sub-endometrial vascular flow index and low IL-15 mRNA are patients with insufficient uNK recruitment and/or inadequate uNK-derived angiogenic-related proteins In contrast, some patients with implantation failures exhibit very high endometrial vascular flow index and at the same time, high IL-15 and IL-18 mRNAs and CD^{56+} cell count. A type 1 immune response could possibly be involved in such profile of implantation failure (Kwak-Kim and Gilman-Sachs, 2008). The number of uNK cells recruited at the time of implantation as well as their state of activation need to be explored in the clinic.

In daily clinical practice, ultrasonic evaluation of subendometrial vascular flow index and measurement of the IL-15 and IL-18 mRNAs together with CD56+ uNK cell counts may thus be useful to identify those women at risk of IVF/ICSI implantation failure.

3. The role of endometrial tumor necrosis factor-like weak inducer of apoptosis (TWEAK)

The role of TWEAK appears very important in implantation. TWEAK is a type-2 transmembrane protein, member of the TNF superfamily. This cytokine is described as acting in a Yin and Yang relationship with TNFα (Bell, 2006), because it counteracts its deleterious effects and has pro angiogenic properties (Donohue et al., 2003). Although it was first described as a weak apoptosis inducer, it triggers multiple cellular responses through its receptor, the fibroblast growth factor inducible-14 (Fn-14). These responses range from proliferation to cell death and stimulation of angiogenesis. It is highly expressed by several types of

immune cells (such as monocytes, dendritic cells, and NK cells) and is expressed in many tissues, including the endometrium. uNK cells expressing the NKp46 cytotoxicity KIR receptor accumulate specifically in the endometrium of patients with concomitant IL-18 over-expression and low TWEAK expression. This suggests that TWEAK is involved in the control of cytotoxicity in uNK cells (Petitbarat et al., 2010).

TWEAK and its receptor Fn-14 localize in the endometrial glands and surface epithelium during the proliferative and luteal phases. They also appear scattered within the stroma, around spiral arteries, and in the luminal epithelium. No differences in immunostaining are detected among the different phases of the cycle, suggesting that TWEAK plays an early role in preventing local cytotoxicity and may counterbalance the cytotoxic function of uNK cells and favor the constructive angiogenic/immunotrophic pathways. Recent studies in humans indicate that the ratio of IL-15 and IL-18 to TWEAK mRNA expression are more important than the normalised values of IL-15, IL-18 or TWEAK mRNA alone (Petitbarat et al., 2010). This testifies that the final balance of controlling signals to activation signals is likely more important than an apparently objective measure of the activation pathways. Indeed, only patients with a high IL-18/TWEAK mRNA ratio quantified by real time PCR show both excessive uNK cell recruitment with simultaneous activation of NKp46, the main endometrial activating receptor of uNK cells cytotoxicity (Petitbarat et al., 2011). In contrast, patient showing low endometrial IL-15/Fn-14 expression exhibit CD56+ uNK cell depletion. Such quantification may therefore be helpful for revealing an imbalance of crucial cytotoxic/angiogenic and immunotrophic pathways.

In summary, the ratios of IL-15/Fn14 and IL-18/TWEAK as quantified by real-time polymerase chain reaction should be evaluated prospectively as 'functional' biomarkers of uterine receptivity to document the mechanisms associated with implantation failures.

4. IL-18/IL-15/TWEAK documentation and personalized strategies to enhance implantation

4.1. Patients with low endometrial angiogenesis and IL-18/TWEAK and IL-15 depletion

In patients showing strong IL-18/TWEAK and IL-15 depletion with an absence of vascularisation, implantation failure could be related to the inability of the endometrium to react to the embryo at the time of apposition and adhesion. Hormonal administration as a consequence of ovarian hyperstimulation will increase the cytokine depletion, since endometrial exposure to a high concentration of oestrogens significantly decreases endometrial IL-18 expression (Ledee et al., 2006). A negative impact of oestrogens on IL-18 expression has been also reported in mice and pigs (Ashworth et al., 2010; Murakami et al., 2005).

In these patients, any strategies leading to the mobilization and activation of endometrial immune cells may be useful. Minimal hormonal stimulation, exposure to the male partner's seminal plasma to enhance immune cell recruitment, local endometrial injury local endometrial injury in the mild-luteal cycle preceding the IVF attempt as well as supplementation with HCG may all improves the potential for implantation. Studies on seminal plasma highlight its role in preparing the uterus for implantation by regulating recruitment and activation of T regulatory cells (Robertson et al., 2009). Local injury in the mid luteal phase of the cycle preceding the IVF/ICSI cycle has been reported to increase pregnancy rates (Almog et al., 2010; Barash et al., 2003; Narvekar et al., 2010). The underlying rationale is that injury causes induction of immune cells and pro-inflammatory cytokines in the endometrium (Gnainsky et al., 2010). The first known human embryo-derived signal, the human chorionic gonadotropin (HCG) profoundly influences immunological tolerance and angiogenesis at the maternal–fetal interface (Perrier d'Hauterive et al., 2007). HCG intervenes in the development of local immune tolerance through apoptosis via Fas/Fas-Ligand. It modulates the Th1/Th2 balance and acts on complement C3 and C4A/B factors influencing decidual immune function. The transient immune tolerance evident during gestation is at least partially achieved via the presence of regulatory T cells which are attracted by HCG into the fetal–maternal interface. HCG treatment of activated dendritic cells results in an up-regulation of MHC class II, IL-10 and indolamine 2,3 dioxygenase (IDO) expression, reducing their ability to stimulate T cell proliferation (Berndt et al., 2009; Tsampalas et al., 2010). Successful implantation also requires extensive endometrial angiogenesis in the implantation site. Recent data demonstrated positive angiogenic effects of HCG via its interaction with endometrial epithelial and endothelial LH/HCG receptors. Particularly, HCG induces VEGF production by endometrial epithelium, increases endothelial cell proliferation and migration of smooth muscle cells leading to the maturation of vessels, an important step for placentation (Berndt et al., 2006). HCG is also involved in the mobilisation of uNK cells via mannose receptor binding (Kane et al., 2009).

4.2. Patients with excessive recruitment of activated uNK cells

In contrast, if patients show excessive recruitment of activated uNK cells, control of the pro-inflammatory environment is essential before embryo transfer. In such conditions, ovarian hyperstimulation as well as a strong supplementation with progesterone (for its immunosuppressive properties) in the mid luteal phase may be useful (Szekeres-Bartho, 2009; Szekeres-Bartho et al., 2009). The place of corticosteroids in such strategies is not clear, with conflicting data on actions in uNK cells. While some authors observed a decrease of uNK cells following corticosteroid administration, others authors reported the opposite. Perez et al. (2005) observed that hydrocortisone is able to enhance the recruitment of CD56 bright cells through IL-15. Corticoids were also found to promote uNK cell differentiation from CD34+ stem cells into mature CD56+ cells (Vitale et al., 2008). Drugs that inhibit the complement pathway, such as heparins as well inhibitors of ocytocin (Moraloglu et al., 2010), need to be evaluated in this context.

To conclude, documentation of the local mobilization of uNK cells as well as their state of activation may be crucial to clinical care. Some patients with a history of embryo implantation failure show an IL-18/IL-15/uNK cell depletion and may fail at the step of embryo apposition because their endometrium is not able to adequately react to support embryo adhesion. Conversely, some other patients with the same history of implantation failure show an excessive uNK cell activation that would lead to failure of the invasion step through excessive cytotoxicity. Randomized cohort studies are now needed to demonstrate that these diagnostic tools will help the clinician in their daily practice, and such trials are mandatory in order to progress.

5. A pre-conception dialogue: follicular G-CSF as a pre-ovulatory biomarker of oocyte competence

Using a microbead-based multiplex sandwich immunoassay (Luminex Technology), we measured simultaneously 27 cytokines and chemokines in each follicular fluid collected from 132 individual follicles of oocytes subsequently fertilized and transferred after conventional ovarian hyperstimulation (Ledee et al., 2008a,b). The originality of the approach was to collect individual follicular fluids and not pooled follicular fluids and to ensure traceability of each sample until birth or failure of the attempt was known. This study showed that the level of G-CSF in individual follicular fluid samples correlates with the implantation potential of the corresponding embryo. The calculation of the area under the ROC (AUC_{ROC}) curve measures the accuracy, i.e. the ability of a factor to discriminate between two distinct outcomes. The following thresholds were used to interpret the AUC_{ROC}: 0.9–1: perfect separation; 0.8–0.9: excellent discrimination; 0.7–0.8: acceptable discrimination; 0.6–0.7: poor discrimination; 0.5–0.6: no discrimination. The AUC_{ROC} performance of embryo morphology as a predictor of implantation ranges between 0.65 and 0.70 for several studies (Guerif et al., 2007). In contrast, for follicular fluid G-CSF the AUC_{ROC} distinguishing the embryos which definitely implanted from those which did not was 0.82 [0.73–0.89] and highly significant ($p = 0.0001$). Significant differences in implantation rates were observed between the embryos with low (<20 pg/ml) and high G-CSF (>24 pg/ml) (9% versus 44% respectively, $p < 0.001$). We subsequently reproduced the data in a cohort of 200 embryos while detailing the adequacy of distinct methods for measuring follicular fluid G-CSF (Ledee et al., 2010). Only the multiplex microbead based technology reach the required sensitivity for adequate quantification.

A third study including 83 women undergoing a modified natural IVF/ICSI cycle was subsequently conducted to provide an experimental model allowing complete traceability, whereby only one oocyte was recovered and only one embryo subsequently transferred (in contrast to the multiple embryo transfer which is conventional in France). Each follicular fluid sample was blindly tested for 26 soluble factors and each mediator evaluated as a potential biomarker of subsequent birth. Reproducibility of follicular composition was evaluated over two modified natural IVF/ICSI cycles for 15 patients. Follicular fluid G-CSF was found to be the best predictor of subsequent birth ($AUC_{ROC} = 0.81$, $p < 0.0001$) when using a multivariate logistic regression model (including known covariates such as age, number of IVF attempts, antral follicle count and embryo quality). The combination of follicular fluid G-CSF and morphological embryo scoring on day 2 increased the AUC_{ROC} to 0.86 ($p < 0.0001$). Birth rates per retrieval were 38% in the group with high G-CSF compared to 5% in the low G-CSF group ($p < 0.0001$). Follicular fluid G-CSF was significantly correlated over two cycles ($r = 0.71$, $p = 0.008$) suggesting the possible prognostic value of its documentation before starting any IVF/ICSI attempt (Ledee et al., 2011). Follicular fluid G-CSF was highly correlated with cytokines IL-7 and IL-17, suggesting key interactions within the follicle involving immune cells such as dendritic cells and regulatory T (Treg) cells. Thus, G-CSF in individual follicular fluids appears to correlate with the birth potential of the corresponding embryo in two distinct models of ovarian monitoring, standard ovarian hyperstimulation and modified natural IVF/ICSI cycles. This finding may have tremendous consequences for the overall morbidity related to reproductive medicine.

At the level of the reproductive tract, G-CSF has been previously shown to be secreted by granulosa cells at ovulation (Salmassi et al., 2004), then within the endometrium during the luteal phase, and finally during gestation in the placenta (Duan, 1990). More recently, a randomized controlled trial reported that G-CSF administration significantly increases live-birth rates in patients with unexplained recurrent miscarriages which failed to respond to a previous immunotherapy treatment (Scarpellini and Sbracia, 2009). Almost all the miscarriages observed in our cohort were found in the group with a low follicular fluid G-CSF level. Follicular fluid G-CSF may promote local maternal–fetal tolerance (Rutella et al., 2005) or influence the oocyte's own mRNA levels or its potential for self-repair (Yannaki et al., 2005). It might also interact with cells in the local microenvironment to induce cytokines and growth factors which are necessary for embryo development and implantation.

6. Contribution of the endometrium to the early cross-talk

Recent studies performed in bovine models were able to demonstrate that the endometrium shows dramatic changes in gene expression related to the way the embryo was produced–by in vivo fertilization, in vitro maturation or cloning (Bauersachs et al., 2009; Mansouri-Attia et al., 2009). In addition, Mansouri-Attia et al. raised the hypothesis that the endometrium could be a natural biosensor for the embryo quality including its potential to develop to term.

In human, an in vitro confrontation model between human decidualised stromal cells and blastocyst suggested the same concept. Decidualised stromal cell gene expression showed significant variations depending on whether the blastocyst was still in development or not (Salker et al., 2010). The result of the study was somewhat surprising, given the main variation in expression indicated a control function aiming to stop the process for non-developing

embryos and suggesting a crucial role of such regulation especially in patients with early miscarriage (Teklenburg et al., 2010). These results emphasize the importance of considering endometrial gene expression during the pre-fertilization and early pregnancy phases in order to evaluate whether the recipient tissue will respond to the embryo adequately.

7. Conclusion

New strategies are emerging to understand the underlying pathways leading to implantation failure and to identify specific defective pathways prior to fertilization and implantation. In this review, we focus on IL-18, IL-15 and TWEAK in the endometrium as well as G-CSF in the ovarian follicle. Identification of new biomarkers attesting to oocyte competence or adequate uterine receptivity should help us to define personalized strategies for infertility treatment. Such strategies may help to improve oocyte fertilization, embryo development and selection, uterine receptivity and ultimately endometrial–embryo cross-talk, an essential process for life.

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