Opinion article

Implantation: can immunological parameters of implantation failure be of interest for pre-eclampsia?

Gerard Chaouat a,*, Natalie Ledee-bataille a,b, Sandrine Zourbas a,c, Sylvie Dubanchet a, Olivier Sandra c, Jacques Martal c, Sasa Ostojojic a,d, René Frydman b

a U131 INSERM, Unite Cytokines dans la Relation Materno-Fetale, 32 Rue des Carnets, 92141 Clamart, France
b Department of Obstetrics/Gynaecology (2), Hospital A Béclère, Clamart, France
c INRA, Jouy en Josas, France
d University of Rijeka, Rijeka, Croatia

Received 28 October 2002; received in revised form 25 November 2002; accepted 26 November 2002

Abstract

We restate briefly why we consider that the Th1/Th2 paradigm, as useful as it has been, is now no longer adequate and is obsolete. We take as an example the role of IL-18, abortifacient at high doses but cardinal for the control of natural killer (NK) cell effects on spiral artery remodelling in mice, and likely also in humans. We then describe briefly our recent studies on cytokine defects and implantation failure in humans, a key feature being the link between uterine cytokine dysregulation and abnormal uterine vascular scores. We draw lessons for pre-eclampsia, and describe features of a model for its immune aetiology.

© 2003 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Cytokines; Networks; Implantation; Paradigm; Human

* Corresponding author.
1. Introduction

Pre-eclampsia is one of the most important obstetrical diseases, being a prevalent worldwide cause of mortality in women at the age of reproduction. Theories on its aetiology are often depicted as two opposing school of thoughts—the “vascularists” for whom oxidative stress results in the disease (Page, 2002; Roberts and Lain, 2002) versus the immunologists who see eclampsia as an autoimmune disease (which it is not) or an alloimmune reaction triggered as a rejection of the fetal allograft, albeit the presence of an oxidative stress and systemic inflammation is now widely recognised. Models exist which combine deficient placentation as a predisposing condition with oxidative stress triggering an abnormally high immune response to the clearance of syncytiotrophoblast debris (Redman and Sargent, 2001).

Recently, epidemiologic studies from Robillard (2002) have further fuelled the immunological concept by introducing the notion of primipaternity versus the classical concept that preeclampsia was a disease of only primiparous women. The argument goes that if preeclampsia is partner-specific, it implies specific recognition which is a key feature of the immune system. However, the fact that preeclampsia is a ‘first pregnancy-only’ disease strongly suggests that a post-delivery mechanism is down-regulating a pregnancy-specific phenomenon. Thus, it suggests that “suppressive” or regulatory memory is involved. This, per se, is indicative of an immune aetiology since only the central nervous and immune systems display memory.

Furthermore, the distinction between vascular and immune events is no longer tenable in view of what is now known of the molecules secreted within the immune system. Most, if not all, cytokines/lymphokines are endowed with pleiotropic properties, amongst which action on the vascular smooth muscle and the vascular endothelium, as well as the coagulation pathway, are the most important for our topic. For example, abortion in a murine resorption-prone system has been linked to activation of fgl2 prothrombinase, the use of knockout mice revealing (to our surprise) that the process was not an attack on the feto-placental unit by natural killer (NK) cells, but instead was abnormal intravascular coagulation (Clark et al., 1998). In that respect, we believe that the recent emphasis put on NK cell recognition of placental antigens, and possible influence of T cell function on NK cells, are the most important emerging concepts in reproductive immunology, with significant consequence for the study of preeclampsia.

The first indication that NK cells could be involved in preeclampsia came from the murine studies of Croy et al. (2001). She observed that uterine NK (uNK)-deficient mice had grossly abnormal placental growth, the placentae
being too small, resulting in an abnormally high rate of in utero fetal loss (Croy et al., 2001). The transfer of NK cells into those NK-deficient mice resulted in correction of placental size and weight, and pregnancy being successfully pursued till normal delivery of healthy pups (Guimond et al., 1998). The key observation was that low placental weight was correlated with abnormal development of local uterine vascularisation (Croy et al., 2001).

These data were somewhat reminiscent of some facets of the “immunotrophism theory” (Wegmann, 1984), except that the immunotrophism precepts were enunciated at a time when T cells were seen as the main component of the immune system and thus as key in the materno-fetal relationship. Yet, this concept and its subsequent demonstration in mice, as well as the studies of Arceci and Pollard on CSF-1 (Bartocci et al., 1986; Arceci et al., 1989), focussed interest on production of cytokines. The studies made during the following decade led to the realisation that the embryo was “bathed in a sea of cytokines”, and led Wegmann also to suggest that “allopregnancy is a Th2 phenomenon, the Th1 cytokines being abortifacient and the Th2 cytokines being protective” (Wegmann et al., 1993). This concept has been tested with success in mice (Lin et al., 1993; Tangri et al., 1994; Chaouat et al., 1995; Krishnan et al., 1996) and humans (Raghupathy, 1997; Raghupathy et al., 1999), albeit almost immediately challenged (Vince and Johnson, 1996). It has been taken as the predominant paradigm to explain the feto-maternal relationship, and used with abuse by some to justify treatment of recurrent spontaneous abortion (RSA) with lymphocyte alloimmunisation.

However, this concept regarding Th1/Th2 cytokines does not fit with the most modern data, be it for established pregnancy or even more for implantation which implies inflammatory cytokines. Indeed, when the concept was initiated, the murine CBA × DBA/2 abortion model appeared to our surprise at the time to be NK cell-mediated (Chaouat, 1986; Clark and Chaouat, 1989), and the results suggested that NK cells could only play a role in rejection of the “fetal allograft”. It took the far-sighted precognition of Y.W. Loke to suggest as early as 1991 (Loke and King, 1991) that the role of NK cells was much more than that and could be both positive and negative, and possibly more important than T cells. Indeed, we know now that uNK cells are crucial, albeit there are discrepancies between the aforementioned studies of Croy and those of the group of Saito (Miyazaki et al., 2002) that remain to be explained. Thus, we know now that they can play either a negative role in abortion, and we initially set up a model of NK cell-mediated resorption to study that facet (Kinsky et al., 1990), or a positive role: as an example of the latter, NK cells, and not T cells, were shown to control IL-10 secretion by the placenta as a consequence of the alloimmunisation process in
the CBA × DBA/2 system (Chaouat et al., 1997). More important even, defects of NK cell knockout mice in the immediate post-implantation period could be due to the fact that NK cells secrete the very important cytokine, angiopoietin 2 (Li et al., 2001). In that context, some kind of NK cell activation could be crucial and, challenging the Th1/Th2 paradigm, it appears that the “bad guy” gamma-interferon, while being abortifacient if injected at high doses, especially in synergy with TNF, is required at lower doses for proper activation of uNK function (Ashkar et al., 2000).

These data have challenged the Th1/Th2 paradigm. Other questioning comes from the spatial distribution of the “new” cytokines (Zourbas et al., 2001), of which IL-18 is a typical example which does not fit into the Th1/Th2 paradigm (Chaouat et al., 2002; Ostojic et al., 2002). Indeed, we find an accumulation of IL-18+ uNK cells during the implantation period, and only then, and only at the implantation point. IL-18 is seen by many as Th1-like, especially in presence of IL-12, which is present in the decidua. It is important to note that it has also Th2-inducing properties in the absence of IL-12 and, in this context, its expression at the implantation period in both stroma and ectoplacental cone is indeed low. It is in fact abortifacient in conjunction with IL-12. In this context, it comes as very important that, when quantifying IL-18 in the CBA × DBA/2 and CBA × BALB/c matings, “we find for IL-18 exactly the opposite pattern as what the Th1/Th2 paradigm will predict” for abortion: “IL-18 production was significantly lower in the decidua from the resorption-prone matings than in the non-abortion-prone ones”, be it for the

Lightycler data IL-18 /GAPDH

<table>
<thead>
<tr>
<th></th>
<th>ABORTIVE CBA/J × DBA/2</th>
<th>NORMAL CBA/J × BALB/c</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEC 9.5 day</td>
<td>72 IL-18 copies</td>
<td>134</td>
</tr>
<tr>
<td>PLAC 9.5 day</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>SPLEEN</td>
<td>81</td>
<td>1246</td>
</tr>
</tbody>
</table>

IL-18 cDNA copies per 100 000 GAPDH copies

Fig. 1. Real-time PCR showing that CBA × BALB/c produce more IL-18 than CBA × DBA/2 matings during pregnancy. The data are pooled from several matings.
decidua or the placenta (Ostojic et al., 2002). These data, obtained by ELISA, have now been confirmed by real-time PCR (Fig. 1).

We believe that such IL-18 patterns, just as for IL-15 (Zourbas et al., 2002) which is a growth factor for uNK but also regulates granzyme content of uNK (Ye et al., 1996; Allen and Nilsen-Hamilton, 1998), can be explained only by a concept which would integrate a dual interaction of NK and T cells (and NKT cells) and place the role of immune cytokines in control of local angiogenesis as central for implantation. We believe that there is an important subset of implantation failure that is immunologically mediated because immune cells do not allow the proper development of spiral arteries via an abnormal inflammation-like reaction. Thus, understanding implantation failure could be very important for preeclampsia.

2. Studies in fertile versus infertile women

Thus, we have now launched a survey of the pre-implantation period in humans, using immunohistochemistry (IHC), flushing, and centred on IL-18 and IL-12, NK counting by an automated software after anti-CD56 labelling, and an echographic evaluation of uterine status with a focus on the uterine vascular status using a common echographic score. We are aware of the fact that many more cytokines will need to be integrated (see below) for a full comprehension of the implantation failures.

We verified first the reproducibility from cycle to cycle of the echographic score as defined in our Hospital, and described by Salle et al. (1998), and indeed it was so ($r = 0.814$, $P = 0.0016$ between 2 cycles) in 15 patients (Table 1a). We then divided the patients in two groups: (a) the implantation failure group comprised women with normal ovarian reserve and no pregnancy, despite more than ten embryos transferred ($n = 37$), and (b) the controls were fertile women followed in pre-natal and pre-implantation diagnosis programmes ($n = 3$) and volunteers ($n = 6$).

The control profile (Fig. 2) was strikingly homogeneous and characteristic (nine individuals): they all showed low glandular or absent IL-12, a neat glandular secretion of IL-18 on the luminal side, and discrete stromal spots of IL-18 secretion around the spiral arteries. Finally, the labelling by anti-CD56 monoclonal antibody of uNK cells yielded low to medium counts. In the patients ($n = 37$), we saw that seven cases were similar to the controls. This is not unexpected since it is inconceivable that all the infertilities may be due only to an IL-12/IL-18-related pathology. Therefore, we believe that these women are representative of those to be explored further by other ELISAs and microarray. In the remaining infertile patients, one can individualise:
Table 1
Evaluation of the uterine arteries score as performed in Béclère Hospital as described by Salle et al. (1998)

<table>
<thead>
<tr>
<th>Score</th>
<th>Follicular phase</th>
<th>Luteal phase</th>
<th>Score (total optimal rating is 20; Salle et al., 1998)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thickness</td>
<td>&gt; 7 mm</td>
<td>&gt; 7 mm</td>
<td>3</td>
</tr>
<tr>
<td>Type</td>
<td>Type 1</td>
<td>Type 1</td>
<td>4</td>
</tr>
<tr>
<td>Homogeneity</td>
<td>Homogeneous</td>
<td>Homogeneous</td>
<td>1</td>
</tr>
<tr>
<td>Index of uterine arterial pulsatility</td>
<td>IPDt + G/2 &lt; 3</td>
<td>IPDt + G/2 &lt; 3</td>
<td>3</td>
</tr>
<tr>
<td>Notch</td>
<td>Absent</td>
<td>Absent</td>
<td>3</td>
</tr>
<tr>
<td>Diastolic flux</td>
<td>Present</td>
<td>Present</td>
<td>4</td>
</tr>
<tr>
<td>Endometrial Flux</td>
<td>Present</td>
<td>Present</td>
<td>3</td>
</tr>
<tr>
<td>Mean ± standard error</td>
<td>Patients</td>
<td>Endometrial thickness</td>
<td>Pulsatility index</td>
</tr>
<tr>
<td>(b) Uterine scores in the pathologic profiles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>10</td>
<td>8.5 ± 0.5</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>If with normal profile</td>
<td>6</td>
<td>9.4 ± 0.2</td>
<td>2.5 ± 0.08</td>
</tr>
<tr>
<td>If with cytokine depletion</td>
<td>16</td>
<td>8.1 ± 1.9</td>
<td>2.7 ± 0.3</td>
</tr>
<tr>
<td>If with cytokine excess</td>
<td>13</td>
<td>8.7 ± 0.3</td>
<td>3.4 ± 0.3</td>
</tr>
</tbody>
</table>

IF: implantation failure.
First profile characterised by an abnormal localisation (stromal instead of glandular) and a high production there (dense IHC labelling) of IL-18 (n = 5).

Second profile showing a high glandular secretion of IL-12, more intense than that of IL-18 (n = 9).

Third profile characterised by high to very high counts of uNK cells (n = 8) showing the various distributions observed, and their dispersion all along the stroma (Fig. 3).

A last profile, which we name total cytokine depletion (n = 8), showing no detectable cytokines at all (it is likely that LIF-deficient women are inside that group).

One can simplify and distinguish two groups by regrouping one as cytokine and NK cell excess, and another as cytokine depletion (Table 1b). We show here only representative pictures for legibility.

Very important for our topic, the abnormal cytokine profiles correlate with vascular anomalies as expressed by vascular score (Table 1b). Thus, the prime determinant of implantation is the proper development of the local vascular

---

![IgG1 isotype control](image1)

![Control group IL-12](image2)

![Control group IL-18](image3)

![Control group CD56](image4)

Fig. 2. Typical normal profiles for IL-12, IL-18 and CD56 endometrial staining (the figure is representative from the control group).
bed, and the localisation of NK cells and their exact activation status (the balance between activation towards a cytotoxic pathway versus a positive cytokine secretion pathway) may be critical in that respect, as suggested by the model of Croy et al. (2001).

### 3. Future prospects

However, the re-questioning of the Th1/Th2 paradigm needs to go further. For implantation, it is clear that further exploration is required, and we believe that this complex network will uncover multiple specific dysregulations which will split the already classified groups into defined molecular defects. The IL-18 studies, as well as other ones conducted in mice and humans, show that precise quantitative measurements are necessary by coupling ELISAs with real-time quantitative PCRs and microarray assays. However, the localisation of the cytokine is also very important, and thus quantitation and localisation need to be coupled using IHC with the help of laser microdissection.

As a first step towards such an approach, we have performed a longitudinal microarray study in CBA × BALB/c versus CBA × DBA/2 mouse matings using the GE array system (Q series kit, Superarray Inc., USA), after a Trizol RNA extraction and DNase treatment to avoid genomic DNA contamination (Invitrogen). Membranes were revealed by a phosphoimager system and analysed at 30 min and 1, 6, 12, 24 and 72 h. We tested the expression of 56 selected genes at various stages throughout...
pregnancy. At all steps, we tested five mice in each group and, for the interpretation, we compared the membranes after normalisation as an N value calculated as: (individual absolute value—“local” membrane background) (sum of values of the 56 spots). We did not use the normalisation process recommended by the manufacturer since it leads to miscalculation of the local background, especially when positive spots are interfering with the local background. We, therefore, used directly the local values determined by the software (AIDA) that was used. In these experiments, we pooled the data from individual chambers or organs.

The results have shown activation of “newer” cytokines, which we had suspected as important, amongst which was the IL-10 family of cytokines (Fickenscher et al., 2002) and, surprisingly, relatively less variation between the two mating combinations than initially anticipated. On the other hand, even though the profiles observed were not as complex as might have been feared, it confirmed the need for further investigation of the cytokine network, while also validating the power of the new technology in the murine

Fig. 4. Day 10 uterine chamber cytokine profiles in a normal vs. abortion-prone murine matings.
system. We show as an example in Fig. 4 the differences between the two mating combinations on day 10 in a full uterine chamber.

4. Lessons for pre eclampsia

With the important caveat of the need for further exploration and dissection of the immune network, the lesson that emerges from the data on implantation is indeed that proper activation of NK cells is important, especially because they accumulate near the arteries and thus might play a key role in their remodelling. Key to the shift towards abortion or preeclampsia would be IL-18 levels and the IL-12/IL-18 ratio.

However, the circuitry might be rather complex, as exemplified by the additional number of cytokines that we are finding to be involved at the fetomaternal interface. With those restrictions in mind, we would like to propose the following model:

- NK cell function is normally linked to secretion of vascular cytokines and is the key regulator of normal spiral artery development.
- Lack of NK cell activation, or activation towards a “cytotoxic pathway” (IL-12 plus IL-18, for example), will result in abnormal NK cell function.
- The activation levels and the regulation levels are different: the abnormal spiral arteries are linked to NK cell dysfunction, whereas suppression induced by regular and repetitive sperm exposure would be at the T cell level.
- NK cell dysfunction could be linked to sudden abnormal recognition of HLA-G and/or abnormal T cell secretion of IL-12, and thus lead to imbalance in the NK cell regulation of angiogenesis.
- Hyperactivation of T cells and NK cells could also lead to local hyperoxidative stress (it is interesting that an IDO deficiency has recently been observed in the placentae of preeclamptic women (Santoso et al., 2002)). It is important also to recall that T cell activation and persistent production of TNF (the Th1 pathway) are known to be involved in recurrent miscarriage and this has once again been confirmed recently in humans as well as oxidative stress linked with miscarriage (Jenkins et al., 2000). In this vein, recently, TNF has been shown to induce oxidative stress in transgenic mice with marked organ-specific alterations in glutathione redox status (Glosli et al., 2002). Thus, immunological pathways and the “vascularist” proposals are not necessarily antagonists.
- Conversely, NK cell downregulation could be exerted via T cell recognition of HLA-C, which would act as a suppressor pathway and be induced by soluble as well as membrane-bound MHC molecules.
Such “suppressive priming” could indeed be done via sperm, involving vaginal/uterine mucosal T cells recognising HLA-C in presence of immunosuppressive/deviating soluble factors in the seminal fluid: this would be a key role for TGF/GM-CSF, as indeed proposed from earlier studies (Robertson et al., 1997, 2002; Robertson and Sharkey, 2001).

In summary, and in agreement with the Croy hypothesis, our recent data point to an important involvement of NK cell regulation in controlling proper uterine vascular flow, well beyond the Th1/Th2 paradigm. Those data are of importance for the treatment of RSA (there is even less logic for treatment of RSA by lymphocyte immunisation than before), for implantation control, and for understanding the aetiology of preeclampsia.

References


