

Rôle du Granulocyte-Colony Stimulating Factor (G-CSF) dans le Processus Implantatoire, chez la Femme et en Modèle Murin "

Mona Rahmati

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ECOLE DOCTORALE BIO-SigNE, ED419

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SCIENCE THESIS IN REPRODUCTION AND DEVELOPMENT

THÈSE DE DOCTORAT SUR TRAVAUX

Public Presentation and Defence on 26th of September 2014 by

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Granulocyte-Colony Stimulating Factor and Embryo Implantation Process:

Effects on Human Endometrium and on Murine Abortion Prone Model CBA/J x DBA/2

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To My Brother

Summary

Reproductive Immunology involves general immunology principles and special aspects of reproduction and development. Colony Stimulating Factors (CSFs) are an illustration of the medical application of this domain. In the CSF family, Granulocyte-Colony Stimulating Factor (G-CSF) appears today as a promising therapy in various cases of reproductive failure although its targets and effects are not clearly established. In this work, through a review on CSFs in reproduction, a study dedicated to human endometrial targets of G-CSF, and a study dedicated to systemic G-CSF supplementation effects on murine embryo implantation, we tried to approach some possible mechanisms of action of this cytokine. In the considered non-abortive abortion-prone murine models, the timed systemic G-CSF and supplementation, targeting specifically the pre implantation endometrium, influenced the embryo implantation process. Some pre conceptual human endometrial dysregulations of G-CSF target genes were also observed in infertile patients. The endometrial influence of G-CSF on these target genes was also illustrated in an ex-vivo model. These molecules under G-CSF influence are described as critically involved in embryo implantation process, by influencing embryo adhesion, cell migration, tissue remodelling and angiogenesis. These data suggest possible pre-conceptual preventive diagnosis of such reproductive failures and future orientated therapies to optimise the endometrial biosensor and the further embryo implantation and ongoing pregnancy.

Key Words

Colony Stimulating Factors, Granulocyte-Colony Stimulating Factor, Reproductive Immunology, Embryo Implantation, Endometrial Biosensor, Human Endometrium, Murine Abortion Prone Model

Résumé

L'immunologie de la reproduction englobe les principes de l'immunologie générale et les aspects spécifiques de la reproduction et du développement. Les Colony Stimulating Factors (CSFs) sont une illustration de l'application médicale de ce domaine. Dans la famille des CSFs, le Granulocyte-Colony Stimulating Factor (G-CSF) apparaît aujourd'hui comme une thérapie innovante dans divers cas d'échec de la reproduction, bien que ses cibles et ses effets ne soient pas encore clairement établis. Dans ce travail, à travers une revue sur les CSFs dans la reproduction, une étude consacrée aux gènes cibles du G-CSF dans l'endomètre humain, et une étude consacrée aux effets de la supplémentation systémique en G-CSF sur l'implantation embryonnaire murine, nous avons essayé d'approcher certains mécanismes d'action possibles pour cette cytokine. Dans les modèles murins fertiles et pro-abortifs, la supplémentation systémique en G-CSF, ciblant spécifiquement l'endomètre préimplantatoire, modifie les taux d'implantation embryonnaire. Dans l'endomètre humain, certaines dérégulations préimplantatoires de gènes cibles du G-CSF ont également été observées chez les patients infertiles. L'influence du G-CSF sur ces gènes cibles a été également illustrée dans un modèle ex-vivo de culture endométriale. Ces cibles dont l'expression est influencée par le G-CSF sont décrites comme des molécules clés dans le processus implantatoire, intervenant sur l'adhésion embryonnaire, la migration cellulaire, le remodelage des tissus et l'angiogenèse locale. Ces données suggèrent des possibilités de diagnostic préventif et pré-conceptionnel de certains échecs de reproduction, considérés jusqu'à maintenant comme idiopathiques, et de thérapies innovantes orientées, afin d'optimiser la réceptivité du biosenseur endométrial afin de permettre une implantation embryonnaire harmonieuse et une grossesse évolutive.

Mots Clés

Colony Stimulating Factors, Granulocyte-Colony Stimulating Factor, Immunologie de la Reproduction, Implantation Embryonnaire, Biosenseur Endométrial, Endomètre Humain, Modèle Murin Pro-Abortif

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List of Abbreviations

ART: Assisted Reproductive Therapy BCR: B Cell Receptor CCL: Chemokine Ligand cDNA: complementary Desoxyribo Nucleic Acid **CSF**: Colony Stimulating Factor CTL: Cytotoxic T Lymphocyte **DAF**: Decay Accelerating Factor DC: Dendritic Cells **DPC**: Day Post Coitum EGF: Epidermal Growth Factor FGF: Fibroblast Growth Factor Fn14: Fibroblast Growth Factor inducible-14 G-CSF: Granulocyte Colony Stimulating Factor **GM-CSF**: Granulocyte Macrophage Colony Stimulating Factor GP130: Glycoprotein 130 HB-EGF: Heparin Binding Epidermal Growth Factor hCG: human Chorionic Gonadotropin HLA: Human Leukocyte Antigen **IDO**: Indoleamine 2,3-dioxygenase **IF**: Implantation Failure IFN: Interferon **IGF**: Insulin Growth Factor IGF-BP-1: Insulin Growth Factor Binding Protein-1 IL: Interleukin

APC: Antigen Presenting Cell

IP: Intra Peritoneal ITGB3: Integrin alpha-v/beta3 **IUGR:** Intra Uterine Growth Restriction **IVF**: In Vitro Fertilization **JAK**: Janus Kinase Family KIR: Killer cell Immunoglobulin-like Receptor KO: Knocked Out LAKC: Lymphokine Activated Killer Cells LH: Luteinizing Hormone LIF: Leukaemia Inhibitory Factor MCP: Monocyte Chemostatic Protein M-CSF: Macrophage Colony Stimulating Factor MHC: Major Histocompatibility Complex MIP: Macrophage Inflammatory Protein mRNA: messenger Ribo Nucleic Acid MUC-1: Mucine-1 **NK**: Natural Killer PDGF: Platelet-Derived Growth Factor **PGE2**: Prostaglandin E2 **PIBF:** Progesterone Induced Blocking Factor PLAUR: Plasminogen Activator Urokinase Receptor **RM**: Repeated Miscarriages **RT**: Reverse Transcription RT-PCR: Real Time Polymerase Chain Reaction STAT: Signal Transducers and Activators of Transcription TCR: T Cell Receptor **TGF**: Transforming Growth Factor Th1/Th2/Th17: T helper cell type 1/2/17

TLR: Toll Like Receptor TNF: Tumour Necrosis Factor Treg: T regulatory lymphocyte TWEAK: TNF WEAK inducer of apoptosis TYMP: Thymidine Phosphorylase uNK: uterine Natural Killer VEGF: Vascular endothelial Growth Factor VEGF-R: Vascular endothelial Growth Factor Receptor

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Preamble



My Medical Thesis was about Colony Stimulating Factors as an example of applied Reproductive Immunology in medicine. During the defence, the President of the Jury, yet amongst the most scientifically involved obstetricians in Paris, asked the first question:

- But, Mona, why choosing immunology?

So, when you're a gynaecologist, your interest towards immunology has to be justified. If only he knew.

How to trap a becoming gynaecologist into reproductive immunology

June 2000. I am a medical student since four years. Final annual results are published in Paris V - Rene Descartes University. I passed "Fundamental and Clinical Immunology" with a "B". I do promise to myself to never ever approach this highly complex, inconstant and unstable field, in any manner and under any circumstances. Surgery seems a more reliable option.

July 2006. I am a specialist registrar since three years and trying from the first day of my nomination in Obstetrics and Gynaecology to get into scientific research, having the prerequisite ready for a master on embryo development. First I was told that I should learn how to perform caesarean sections and hysterectomies - which was done - and then, that the field seemed specifically dedicated to biologists, geneticists or pathologists - which was more frustrating -.

Beginning of July, a very famous foetal medicine congress takes place in our department, and being the youngest registrar, I have the honour to be continuously on call for a couple of days. The last morning, in a particularly good mood, I meet the senior on call for emergencies. I never met her before. Apparently she's the youngest consultant in reproductive medicine, Nathalie Ledee, who has had the same honour than me. Given my exceptional mood during the first caesarean section, she tries to understand what could be wrong and I explain her that any innovative field seems to be dedicated to older registrars or simply to non-gynaecologists. And she straight tells me that she can accept master students in her lab:

- Well, it's not just development, it's about reproduction and development... With very innovative aspects, of course... Working in a European network of excellence... We should organise your research master for next year... Well, the university deadline was yesterday, but we should be able to arrange...

During the second caesarean section, she has a long non-understandable monologue, most probably explaining the subject of her researches. And when noticing my absence of reactivity, she asks:

- Ok, now, do you know endometrial arteries being invaded by trophoblast during placentation?

- Yes.

- Ok, then, you're going to work on these arteries before their invasion... What's your e-mail address?

A few weeks later, I am leaving her office with a pile of thick books and congress reports:

- By the way, you have to meet Gerard Chaouat. Here is Marie Petitbarat's e-mail address, she will tell you where and when. Don't be impressed and don't be afraid if you don't understand anything the first time you listen to him. Sylvie Dubanchet will be next to him, she'll explain you later.

And while coming out, she gives me an extra article from the amount of papers on her desk, by a Japanese guy talking about Th17... I vaguely remember some Th-something somewhere in my studies, Th1 or 2 and certainly not 17. But where was it then? Oh no! That's it, I'm trapped. And once you're trapped, your interest towards reproductive immunology can't be punctual, you get addicted.

September 2008. After the year of research master, Nathalie Ledee calls me about some delayed articles, presentations and posters:

- It was a nice subject. You should follow with a Science Ph.D. We should try for next year.

- You know, I'll need to take at least two years off from hospital. I'm not sure they'll propose me the same position after a two years' gap. I don't have any specific funding for a thesis project. And I already told you, I can't fiddle mice, I have a phobia.

- I understand. I have a serious plan in mind. The submission deadline was yesterday, but we should be able to arrange.

And her calling me a few weeks later: "Yes, We Can!"

Reproductive immunology today

Immunology today is a widespread field, involved in any medical sub-speciality. Reproductive immunology is a specific domain, involving general immunology principles and special aspects of reproduction and development. A short review of the history of reproductive immunology is suggested in a further chapter.

Some independent international societies are exclusively dedicated to reproductive immunology, such as International Society for Immunology of Reproduction (ISIR), European Society for Reproductive Immunology (ESRI) or American Society for Reproductive Immunology (ASRI). And many other active groups are within national societies for immunology.

Two internationally admitted journals are specifically dedicated to reproductive immunology: the Journal of Reproductive Immunology (JRI) and the American Journal of Immunology (AJRI).

And ultimately, reproductive immunology found its new Holy Book, actually a holy e-book. Gerard Chaouat's Little Red E-Book, entitled "Immunology of Pregnancy" is co-edited by Nathalie Ledee and Olivier Sandra. This book is not only a collection of quotations from our supreme immunological guide, but the most updated collected data, written by the major international specialists of each field. Given the very active evolution of immunological concepts, I do advise you to hurry in getting and reading it.

Why trying to attract gynaecologists towards reproductive immunology

Once the fundamental part fulfilled, the last dam to pass in reproductive immunology is communicating with the gynaecologists. It seems so unfair, when other fundamental immunologists have to deal with rheumatologists, dermatologists or haematologists who are amongst the most receptive people in medical field, reproductive immunologists have to cope with the most inaccessible, delayed, impatient, rushed, sleepless and sometimes aggressive people in hospitals.

Nevertheless, explaining the interest of reproductive immunology to gynaecologists is vital... And it's vital for reproductive immunologists. Gynaecologists are the last link in this chain, applying the very precious fundamentally demonstrated principles. Knowing the rational of some pathologies may help in improving infertility treatment or a better obstetrical management. But being aware of such fundamental data may also prevent from inadequate, unsubstantial or even deleterious use of so called immunological treatments.

Moreover, gynaecologists are generally more reluctant to new approaches than other specialists, given some sensitive aspects of their field, particularly the risks linked to embryonic or foetal toxicity, and high legal pressures. Introducing reproductive immunology during their training might be helpful for considering further innovative approaches during their profession.

I was lucky enough, in my personal curriculum, to meet exceptional people who dared to come to my level and adapt their speech to make me discover their interests. I hope I can transmit their knowledge and arouse more interest around me.

Some people I would like to thank

I thank Dr Nathalie Ledee for orienting me towards this fascinating field, suggesting this interesting subject and innovative approaches. Thank you for your constant guidance, support, availability and encouragement.

I thank Dr Gerard Chaouat for this initiation to reproductive immunology, for adapting his knowledge to my level and for the enjoyable co-direction of this work.

I thank Mrs Sylvie Dubanchet and Mrs Marie Petitbarat for their invaluable permanent technical help and friendship during these years.

I thank you all for having accepted me as part of your team and sharing your knowledge as well as your material and technical means. I do not consider these acknowledgements as an end, but as a start to further collaborations.

I thank Pr Armand Bensussan and Dr Jean-Yves Picard for hosting this work in their research units.

I thank Dr Elisabeth Menu and Dr Pascale Chavatte-Palmer for tutoring this work, defining its limits and refocusing it on the main subject during its elaboration.

I thank Dr Elisabeth Menu and Dr Celine Mehats for their prompt and constructive reports.

I thank the President of the Jury, Pr Michael Schumacher, and all the Jury Members, Dr Elisabeth Menu, Dr Celine Mehats, Pr Michael Summers, Dr Richard Balet and Dr Olivier Sandra, for their availability and interest.

I thank my latest clinical heads for accepting to adapt my clinical time to fulfil this fundamental project: Pr Marc Dommergues, Dr Richad Balet and Dr Sophie Gaudu.

I thank particularly my parents for their constant support in all ways during all these years, helping me to accomplish my multiple projects. And I thank my husband and children for accepting this unusual life rhythm.

I finally and specially thank my brother, to whom I dedicate this work, for his perpetual help and guidance since ever, allowing me to achieve whatever I want and plan in a much easier and prepared manner.

1. Introduction

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1.3. Hypothesis and Objectives

1. Introduction

1.1. Reminder on Embryo Implantation

1.1.1. Overview of embryo implantation steps

Despite more than 30 years of remarkable progress in Assisted Reproductive Therapies [1], the embryo implantation remains a black box period, and its study in situ remains impossible in Human, given obvious ethical reasons. Still, this is the putative chronology of the early course of the embryo.

In Human, after a natural fecundation and a ride through the fallopian tube, the embryo reaches the uterine cavity at day 5. The embryo at this time is at the stage of blastocyst, consisting of an inner cell mass enclosed in a trophectoderm shell. After hatching, i.e. emerging from its surrounding zona pellucida, the blastocyst will start its anchoring to a specifically prepared endometrium (Figure 1).

Figure 1: **Overview of Human embryo implantation steps** From Fitzgerald, Human Reprod Update, 2008



Apposition and adhesion are the two first events of the blastocyst attachment to a receptive endometrium. Then the extensive but very tightly controlled process of invasion takes place, to allow the further placentation [2].

The apposition, adhesion and invasion corresponding mechanisms will be detailed in the following paragraphs, together with differentiation of first the ectoplacental cone and then organisation of trophoblast, e.g. placenta, and delimitation of annexes.

The embryo implantation is a highly invasive process, comparable to neoplasia dissemination, which could take place on any organ where the placental vascular bed can spread. Various case reports have already described invasive ectopic pregnancies on peritoneum, spleen, liver, bowel or kidney [2].



Figure 2: Chronology of uterine events in Human

P.O. days: Post Ovulatory days **LH**: Luteinizing Hormone

Implantation can occur on any vascularized tissue, but endometrium. During the majority of the endometrial cycle, the embryo will not be able to interact with the endometrium, except during a specific period of five days called the window of implantation (Figure 2).

1.1.2. Endometrial metamorphosis

The window of implantation lasts from day 5 to day 9 after ovulation and progesterone secretion from corpus luteum [3]. Specific endometrial changes occurring during this period will define the receptive endometrium.

Structural remodelling affects endometrial surface epithelium, endometrial glands, endometrial stroma and endometrial and sub endometrial arteries. This transformation is called endometrial decidualisation and takes place independently from the presence of the embryo (Figure 3). The surface of luminal epithelium increases by emergence of apical pinopodes and microvilli. Endometrial glands turn spiral and secretory. Stromal fibroblast-like cells become large and round. The stromal extra cellular matrix becomes looser and changes its composition [4]. Decidualisation also includes a deep remodelling of endometrial spiral arteries, with endothelial swelling, vacuolisation and vascular smooth muscle disorganisation, essential to a further trophoblast invasion [5]. In Human, this pre implantation decidualisation occurs every cycle, independently from the presence of the embryo itself [4].

Figure 3: **Histological slides of proliferative (a) and secretory (b) endometrium** Slides from paraffin embedded samples, Haematoxylin and Eosin Staining (x400)



G: Endometrial Glands S: Endometrial Stroma A: Endometrial Artery At molecular level, the expression and the secretion of various factors underlie the structural endometrial changes. Some specific molecules enable the anchoring process which takes place during the apposition and adhesion steps. The glycocalix which normally recovers the endometrial apex, to turn it anti-adhesive and resistant to microbial attacks, disappears [6]. L-selectin oligosaccharide-based ligands production by endometrial epithelium is up-regulated during the window of implantation, as first receptors to L-selectins expressed on the blastocyst [7] [8]. Tighter anchorage of the blastocyst is allowed by a specific pattern of integrins expression, forming focal adhesion sites on endometrium. Integrins are expressed on both endometrium and blastocyst. Through some extra cellular bridging ligands and the recruitment of a network of cytoskeletal proteins and intracellular signalling complexes, integrins are supposed to mediate cellular adhesion and migration [9]. The swelling extra cellular matrix in receptive endometrium is rich in collagen, fibronectin and laminin, as part of the cellular adhesion and migration system [4].

These expressions are under cyclic regulation of ovarian hormones. Estrogen enhances cell proliferation in uterine epithelium and prepares the specific decidualisation by inducing the local progesterone receptor expression. Progesterone, the essential hormone for embryo implantation and pregnancy maintenance in mammals, has a key role in proliferation and differentiation of stromal, glandular and myometrial cells [10] [11] [12], as well as in the local immune modulation [13] [14] [15]. Further paracrine and embryonic factors help also in creating a transient primary inflammatory reaction followed by a tolerant immune environment necessary to a successful embryo implantation and they will be detailed in the corresponding paragraphs.

Endometrial decidualisation is finally characterised by a local immune switch, from an adaptative immunity protecting the endometrium against infections, to a specific innate immunity, allowing the implantation of the semi-allogenic embryo. The endometrial immune cell population during the implantation window includes uterine Natural Killers cells (uNK), macrophages, regulatory T cells and dendritic cells [16]. The properties of each type of these cells will be detailed further. The majority of this immune population consists of uNK cells [17]. These specific cells do not only take part in the local immune modulation, but above all, promote the spiral arteries remodelling which has already been described [18] through specific secretion of cytokines and angiogenic factors [19] such as, Vascular

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Endothelial Growth Factor (VEGF), Angiotensin I and II, Interferon gamma (IFNγ) and Nitric Oxide (NO) [20].

1.1.3. Dialogue between mother and conceptus

The decidualised endometrium is described as a biosensor [21] able to establish a dialogue with the coming blastocyst. On one side, the endometrium has to be receptive to start



From Sempe, Le Petit Nicolas, 1960

interacting with the embryo, but on the other side, signals from the blastocyst can modulate the maternal answer towards implantation or rejection.

To start the overview of the very delicate, complex and stage specific local equilibrium which is involved in the tissue remodelling that controls uterine receptivity and establishes a mother-conceptus dialogue [22], we can simplify the process by splitting it into two phases: first, a transient pseudo-

inflammatory reaction followed by the establishment of local immune modulation.

Schematically, the first inflammatory step in the receptive endometrium, Th1 immunity dominated, is needed for endometrial destabilisation to fulfil the apposition and adhesion process. Endometrial presence of inflammatory cytokines such as Interferon gamma (IFN Υ),Tumor Necrosis Factor alpha (TNF α), Interleukins 1, 2 and 6 (IL-1, IL-2, IL-6) or Leukaemia Inhibitory Factor (LIF) enables the anchoring and the cell mobilisation systems for the embryo attachment [23]. During this initial phase the vascular tripod formed by Interleukins 12, 15 and 18 (IL-12, IL-15, IL-18) is essential for the endometrial vascular remodelling through the local control of uNK cells recruitment and activation [24].

Then a local immune modulation, called switch to a Th2 dominated profile, is required to avoid embryo rejection as non self after cell mediated killing and lysis by the endometrial immune cells. Amongst local cytokines, Transforming Growth Factor beta (TGFβ) or

Interleukins 4 and 10 (IL-4, IL-10) are described as Th2 cytokines [25]. With this specific environment, endometrial immune cells divert from their cytotoxic capacity whereas uNK cells do not acquire a cytotoxic phenotype, preventing embryo recognition and lysis, while maintaining their trophic and angiogenic factor producer status, helping to maintain embryo invasion and growth [26].

This local immune modulation can be enhanced by the embryo itself. First, the embryo modulates the immune endometrial response by producing several immune suppressive factors such as Indoleamine 2,3-Dioxygenase (IDO) [27], Prostaglandin E2 (PGE2) [28], anti-complement molecules such as Monocyte Chemostatic Protein (MCP) and Decay Accelerating Factor (DAF) [29], and placental microparticles as exosomes [30] [31].

The embryo also enhances this local immune modulation by its specific antigenicity. Trophoblast cells are free of Major Histocompatibility Complex (MHC) Class II (by a phenomenon of hypermethylation) [32], and free of MHC I polymorphic molecules such as Human Leucocyte Antigen (HLA) A or B thus avoiding maternal attacks from cytotoxic T cells. Trophoblastic cells nevertheless express some molecules of the non classical MHC class I monomorphic G and E and the limited polymorphic HLA C, thus limiting maternal attacks from cytotoxic T cells which usually kill non-self cell, while the uterine natural killer KIR system is defusing uNK by expression of HLA-C and on invading extravillous cytotrophoblast HLA-G (see below). Some specific placental components, such as syncytiotrophoblast and extra-villous cytotrophoblast, which are directly in contact with maternal structures, also secrete soluble forms of HLA-G [33] [34] [35]. Both membrane and soluble HLA-G decrease lytic activity of uNK cells and turn their secretions into trophic and angiogenic ones. Soluble HLA-G also stimulates the apoptosis of the local maternal activated Cytotoxic T Lymphocytes (CTL).

When entering the uterine cavity, blastocyst is also shown to be able, via cytokine production of trophectodermic, and later on, for the embryo proper, trophoblastic cells, to modulate endometrial receptivity through regulation of the expression of various adhesion molecules. For example, in vitro models have shown that embryonic secretions, specially Interleukin 1 (IL-1) system, may help the attachment to the endometrial epithelium by

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enhancing and clustering pro-adhesion membrane molecules such as integrins [36] [37] or by down regulating production of anti-adhesive surface molecules such as mucins [38].

Human Chorionic Gonadotropin (hCG) is another example of embryonic action on the endometrium to facilitate implantation. This glycosylated hormone is mainly produced by syncytiotrophoblast. It stimulates endometrial decidualisation, modulates secretion of Th1 cytokines such as LIF [39] and enhances production of angiogenic factors such as Vascular Endothelial Growth Factor [40] [41]. It also has been demonstrated that hCG influences the recruitment and proliferation of surrounding maternal NK cells [42].

An illustration of this mother-conceptus dialogue, strengthening the hypothesis of the receptive endometrium being a biosensor towards the embryo, was shown in an in vitro confrontation model [43] [44]. Gene expressions varied between decidualised stromal cells in contact with developing blastocysts or non-developing blastocysts. The variation in the decidual expression was orientated towards inhibition of implantation process for non-developing embryos. Such results emphasize the importance of the pre-implantation embryo ability to respond to a receptive endometrium.

These mutual repeated mother-conceptus interactions are essential to enhance embryo implantation but also to control further embryo invasion.

1.1.4. Placentation process

After the adhesion of the blastocyst to maternal endometrium, and the creation of the ectoplacental cone, trophoblast cells differentiate into the outer syncytiotrophoblast and the inner cytotrophoblast. The blastocyst embedding into the endometrial stroma starts with the trophoblast ability to degrade the extra cellular matrix through production of lytic enzymes and triggering apoptosis of endometrial cells. This tissue erosion also affects surrounding capillaries, establishing the first utero-placental circulation system. This process of early embryo implantation ends by the second week of development.

During the third week of gestation, once embryo embedding in the endometrial stroma is completed, primary, secondary and tertiary villi are shaped. Primary villi are formed by penetration of cytotrophoblast into syncytiotrophoblast. Secondary villi are formed by the infiltration of extra-embryonic mesoblast into the primary villi (Figure 4). And the tertiary villi contain blood vessels originated after differentiation from the extra-embryonic mesoblast.

Figure 4: Early steps of Human placentation

From Cummings, Pearson Education, 2004



Among the tertiary villi, some will reach the endometrial basal plate, forming the anchoring placental villi by rapid proliferation of cytotrophoblast cells. From these proliferative cells, some highly invasive extra-villous trophoblast cells will start their migration. The extra-villous trophoblast cells can be grouped into two categories [45]: interstitial cytotrophoblast cells invading the endometrium and the proximal third of the myometrium, and the endovascular cytotrophoblast cells, invading and remodelling the decidualised uterine spiral arteries. This process will lead to the establishment of a low resistance vascular system, allowing increased arterial blood flow and facilitating maternal-foetal exchanges in nutrient and gas (Figure 5).

Figure 5: Human trophoblast populations in the first trimester of pregnancy

From Ashley Moffett-King, Nature Reviews Immunology, 2002



a. The placental villi are covered by villous traphoblast cells: an inner mononuclear cytotrophoblast layer covered by syncytiotrophoblast. The core of each villus contains fetal blood vessels, fibroblasts and fetal macrophages (Hofbauer cells). Maternal blood in the intervillous space reaches the placenta through the uterine spiral arteries (A). The inner layer of villous cytotrophobiast grows out at facal points to form cell columns (CT). These are prominent at the anchoring villi, where attachment to the maternal decidua occurs. At the fetal-maternal boundary, the columns form a partially continuous shell. From this shell, extravillous trophoblast cells enter the decidua as interstitial trophoblast (T) to encircle and destroy the arterial media, which is replaced by fibrinaid material (F). Then, endovascular trophoblast cells (E) move down the arteries in a retrograde manner to replace the endothelial cells. The trophoblast cells move as far as the inner myometrium, where they fuse to become placental-bed giant cells (GC).

b. A schematic representation of the decidual stroma at the implantation site. Interstitial traphablast cells (T) are seen between large stromal cells (S). The maternal leukocytes that are present are uterine natural killer cells (K), as well as a few macrophages (M) and occasional T cells (L).

The previously described process in Humans leads to a hemochorial placenta. In mice, placentation is also hemochorial with trophoblast migration and arterial remodelling to a lesser extent (Figure 6).

In this type of placentation, foetal structures, like syncytiotrophoblast in Humans, are directly exposed to maternal blood elements such as leukocytes.

Figure 6: Decidualization and placentation in mice

From Lim, J Clin Invest, 2010

M: Mesometrial Side IS: Implantation Site

On day 4 On day 7 On day 13 The luminal epithelium closes on an The embryo is much larger, and its The placenta has developed, *implanting blastocyst. The mural* ectoplacental cone has penetrated and the decidua has regressed trophectoderm, which is distant from the mesometrial decidua. to thin layers around the inner cell mass, contacts the Differentiated decidual cells now take the placenta and embryo, epithelium at the antimesometrial up most of the implantation site. known as side. The decidual response also Decidualization does not occur at decidua basalis and decidua capsularis, respectively. starts from the antimesometrial side. inter-implantation sites. AM: Anti Mesometriai Side

In mice, where labyrinths form equivalent structures to placental villi in Humans, the outermost labyrinth trophoblast cells are in direct contact with the maternal blood. Human cytotrophoblast cells, equivalent to murine trophoblast giant cells, are also in close association with maternal decidual leukocytes [46] [47]. These similarities in structure and maternal-foetal immune confrontation, suggest that mice might be a fairly close study model in this field (Figure 7).

With this highly invasive placentation, a large contact surface occurs between foetal and maternal structures, permitting an accommodation to the progressive needs of the growing foetus. A defect in this process may then cause pathological consequences.

Figure 7: Comparative anatomy of human and mouse placentas

From Maltepe, J Clin Invest, 2010



In the hemochorial placentas of humans (A-C) and mice (D-F), the maternal vessels are invaded and colonized by invasive trophoblasts (not shown).

(A) Consequently, in humans, decidual spiral arterioles perfuse the chorionic villi that line the intervillous space.

(B) In floating villi (FV), a continuous layer of multinucleated SynTs (syncytiotrophoblasts)interfaces with maternal blood. Beneath lies a progenitor population of mononuclear vCTBs (villous cytotrophoblasts). At the uterine wall, iCTBs (invasive cytotrophoblasts) differentiate along the invasive pathway to form anchoring villi (AV). A subset of iCTBs breaches spiral arterioles and differentiates into an endovascular subtype that replaces the resident maternal endothelium (not shown).

(C) The cross-sectional anatomy of a floating villus shows that the apical surfaces of SynTs are covered with branched microvilli that maximize their surface areas for gas and nutrient/waste exchange. The blood vessels that ramify through the villous stroma carry embryonic/fetal blood.

(D) In mice, maternal blood from decidual spiral arterioles flows through blood sinuses in the SpT (spongiotrophoblast) layer to reach the labyrinth.

(E) TGCs, like iCTBs, anchor the placenta to the uterus and invade the spiral arterioles (not shown).

(F) In mice, maternal blood is in direct contact with a layer of mononuclear trophoblasts (MNTs) also known as sinusoidal trophoblast giant cells (S-TGCs) that is surrounded by a bilayer of SynTs, which are in close proximity to fetal capillaries.

1.1.5. And when something goes wrong ...

From the endometrial point of view, a defect in decidualisation and receptivity, a local immune hyper activation or a local disruption may induce various pathologies.

When considering early pregnancy, a local immune dysregulation may results in clinical conditions such as early miscarriage (when the embryo implantation starts but the pregnancy spontaneously terminates during the first trimester) or embryo implantation failure (when the embryo implantation process does not even start).

An insufficient initial inflammatory reaction will not help the emergence of endometrial anchoring elements leading to an absence of embryo apposition and adhesion steps.

On the other hand, an uncontrolled initial endometrial inflammatory reaction, particularly by turning local Natural Killer cells cytotoxic, will enhance the recognition of the embryo as non-self, stop the invasion process and provoke embryo lysis [48].

To a lesser extent, the limitation of the invasion process by disequilibrium in the local immune balance, without leading to initial destruction of the embryo, will result in further obstetrical complications through placental pathologies.

An insufficient embryo invasion and specially an inadequate remodelling of the spiral arteries, either by a lack of initial decidualisation or by further trophobast destruction, will lead to a poor trophoblast invasion and limited placental function.

This may result in clinical conditions such as intrauterine growth restriction (IUGR) or preeclampsia (Figure 8), a complex and multifactorial pathology which affects 5% of all pregnancies and initially manifests with maternal hypertension and proteinuria [49].

Figure 8: Normal and pathological Human trophoblast invasion and consequences

From Ashley Moffett-King, Nature Reviews Immunology, 2002



A comparison between:

- uninvaded arteries (non-pregnant)

normal pregnancy

 pathological conditions of pregnancy such as pre-eclampsia and introuterine growth retardation (IUGR) —

The extent and depth of trophoblast invasion is less in pathological compared with normal pregnancy, which results in Inadequate transformation of the spiral arteries in the former.

This results in reduced blood flow to the feto-placental unit, which leads to poor fetal growth.

The ischaemic placenta can also trigger systemic endothelial dysfunction in the mother and the anset of pre-eclampsia.

Mechanical endometrial disruption consequences also points out the local influence on placental regulation. On previous uterine scars, after caesarean section or myomectomy, where endometrial barrier has been altered and the control of the invasive potential of the trophoblast could not be limited, we observe a higher incidence of uncontrolled placental invasion, leading to placenta accreta [50]. Placenta accreta occurs by a morbid placental adherence to the uterus. It is divided into three grades based on histopathology: placenta accreta where the chorionic villi are in contact with the myometrium, placenta increta where the chorionic villi are in contact with the myometrium, placenta increta where the uterine serosa. This severe pregnancy complication is an important cause of maternal morbidity and mortality, specifically through a high risk of [51].

1.2. Reminder on Reproductive Immunology

1.2.1. Reminder on general immunology

When simplified to the extreme, immunology deals with t self and non-self recognition, to ensure own defence and integrity. It is classically divided into innate immunity and adaptative immunity, either humoral or cell mediated. Innate immunity concerns mainly continuous, pre-existing recognition of non-self molecules in microorganisms and changes in self molecules or lack of self molecules on cells altered by infection or neoplasia for example. Adaptative immunity is based on self and non-self distinction via generation of large specific receptors repertories recognizing non-self antigens. Cells presenting high avidity receptors toward self molecules have also to be eliminated or inactivated by adaptative immune elements. An imperfection on self and non-self discrimination leads to autoimmune pathologies. Given the enormous variety of antigens and immune receptors, the difference between self and nonself is not absolute. It depends on thresholds of activation, which define the extent of self and nonself discrimination limits and immune responsiveness [52] [53].

Innate immunity includes antigen non-specific elements capable of immediate response to pathogen attacks, within minutes or hours. Apart from physical barriers such as skin or mucous membranes and chemical barrier of pH, innate immunity actors are leukocytes, other than B or T lymphocytes, such as macrophages, dendritic cells, neutrophils and Natural Killer cells [54]. These cells form the first defence against pathogens invasion, for example through Toll-Like Receptors (TLR), recognising pathogen-associated molecular patterns (PAMP) found on microorganisms [55] [56]. Another function of this system is regulating adaptative immunity through cytokines production and assisting antigen presentation [54].

Adaptative immunity manages specific defence and ensures immune memory in case of reexposure. These functions require steps of antigen presentation, effective response and memorisation. The first step is fulfilled by Antigen Presenting Cells (APC), such as macrophages or dendritic cells, which will process antigens for presentation to T cells, to induce effector lymphocytes activation. Effector functions consist in specific antibody production called humoral immunity, performed by B lymphocytes presenting membrane B Cell Receptors (BCR), and in cytokine production and cell destruction called cell mediated immunity, performed by T lymphocytes presenting membrane T Cell Receptors (TCR). We differentiate two subgroups of T cells given their surface proteins: CD4(+) helper cells involved in inducing production of antibodies as well as activation of the CD8(+) cytotoxic cells, themselves involved in cell destruction, also called Cytotoxic T Lymphocytes (CTL) when activated. Finally, immune memory function is provided by memory B cells created during clonal expansions, ensuring a faster specific response in case of antigen re-exposure [52] [53], as well as memory T cells

The T lymphocytes activation happens via recognition of antigens fragments, as peptides presented by Major Histocompatibility Complexes molecules I and II (MHC I and II). MHC molecules are also called HLA in Human (Human Leucocyte Antigen). These heterodimeric transmembrane glycoproteins are divided into two classes, MHC I and II. MHC I molecules are on all somatic nucleated cells. They include classic isoforms HLA-A, B, C and non-classic isoforms HLA-E, F, G. MHC I molecules present peptides, driven from intracellular digestion, to cytotoxic CD8 T lymphocytes. HLA-A and B isoforms are highly polymorphic, unlike HLA-C which has a restricted polymorphism and non-classic isoforms which are nearly monomorphic. Given these properties, as well of course as their selective expression, HLA-C and HLA-G are the major actors of the transient tolerance towards the foetus. MHC II molecules are on immune cells presenting processed antigens to helper CD4 T cells. In Human, there are three different isotypes (HLA-DR, HLA-DQ, HLA-DP), characterized by a very high level of polymorphism. MHC II molecules functions consist in the presentation of exogenous and endogenous peptides to the TCR of CD4 T cells for adaptative immune response, the establishment of the TCR repertoire of the CD4 T cell population through selective events in thymus, and finally the regulation of peripheral CD4 cells lifespan. [57].

All these immune actions are coordinated by secreted soluble factors such as chemokines, which regulate the immune cells attraction, and cytokines, which are the communication tools between the different immune cells. The cytokinic milieu is crucial for the differentiation of immune cells towards a tolerogenic or inflammatory response. Th1 / Th2 / Th17 type immune responses are named after the corresponding CD4 T helper lymphocytes,

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classified from a functional point of view in different lineages. Through the dominance of specific cytokine production and cellular orientation, the milieu can turn inflammatory (Th1) or tolerogenic (Th2) [58]. Recently, the reversibility of these phenotypes has been suggested, the shift between tolerogenic to inflammatory dominant immune responses being mediated by Th17 lymphocytes. Th17 lymphocytes possess a functional plasticity allowing them to shift towards the Th1 or the Th2 phenotype in the presence of modulatory cytokines [59].

Some attempts of mathematical modelisation of these highly complex cellular and cytokinic networks are described. With all the redundancies, feedbacks, multi functionality, plasticity and multiplicity of possible combinations in immunity, mathematically modelising the system would be similar to study a chaotic behaviour [60]. Thus, we will not try to draw up an exhaustive report of the current knowledge on the immune system, but, after a brief historical reminder, will try to present some selected major cytokinic or cellular actors intervening in reproductive immunology.

1.2.2. History of reproductive immunology

Reproductive immunology is a domain involving general immunology principles and specific aspects of reproduction and development. From the classical immunology point of view, embryo implantation and pregnancy are exceptional events. The survival and the growth of the semi-allogenic foetus cannot be explained by usually admitted mechanisms of transplantation.

Albeit Reproductive Immunology started as early as the 19th century with the discovery of immunisation against sperm antigens by Landsteiner and Metalnikoff, but as far as pregnancy is concerned, it really started in 1953. Medawar enunciated "the immune paradox of pregnancy" and suggested the first hypotheses to explain this situation, by invoking physical separation or constraint of the immune response in pregnancy [61]. In 1970's, the first attempt to explain a state of immunosuppression, called "the facilitation theory", emphasized on systemic immune regulation via maternal antibodies hiding paternal placental antigens [62]. But this theory revealed insufficient, as maternal pre-

immunisation or absence of maternal anti-paternal antibodies, in Human or mice, did not impair pregnancy [63]. From the 1980's, study of local regulatory mechanisms proved more productive.

The initially analysed local mechanism was immunosuppression. On one hand, this immunosuppression was demonstrated to be due to locally secreted factors or particles. On the maternal side this consisted in Transforming Growth Factor β (TGF β) or Progesterone Induced Blocking Factor (PIBF) [64] [65], and on foetal side, immune modulators like Indoleamine 2,3-Dioxygenase (IDO) [27], Prostaglandin E2 (PGE2) [28], anti-complement molecules as Monocyte Chemostatic Protein (MCP) or Decay Accelerating Factor (DAF) [29], and placental microparticles as exosomes [31] [30]. On the other hand, it was demonstrated that local immune suppression was allowed given a very specific antigenicity on foetal structures in contact with maternal interface, specially due to absence of highly polymorphic surface antigens, Human Leucocyte Antigen (HLA) A or B, and presence of HLA-C [34] and HLA-G [66].

In parallel, Loke and Croy demonstrated the importance of a specific local immune cell population called uterine Natural Killers (uNK) in embryo implantation process [67]. These specific cells were shown to be essential for the local vascular remodelling and thus allowing an extensive placentation, via cytokine expression and further interaction with foetal HLA-C [34].

When considering cytokines at the maternal foetal interface, Wegmann raised the theory of "immunotrophism" [68]. It was demonstrated that, apart from their local immune function, some cytokines secreted by stimulated maternal CD4 T lymphocytes, had a trophic action on placental and embryo growth. The Colony Stimulating Factors action partly belongs to this field.

At the end of 1980's, T helper lymphocytes and their cytokines were categorized in two groups: Th1 for the cytotoxic and pro-inflammatory ones, and Th2 for the immunomodulatory and anti-inflammatory ones [69]. The same distinction was applied to the cytokines at the maternal foetal interface, and a successful embryo implantation and an ongoing pregnancy were assimilated to a Th2 phenomenon, protecting the conceptus against maternal rejection. Shortly after, the endometrial presence of inflammation actors

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was shown [70] and it was demonstrated that a lack of some inflammatory molecules would impair embryo implantation [71]. An initial transient Th1 reaction, followed by a Th2 environment, was fundamental to enable an effective embryo attachment and a further sufficient embryo invasion without being rejected [25]. This was designated as the "Th1-Th2 paradigm".

With the evolution of the knowledge in general immunology, soon this paradigm appeared too simplistic to explain pregnancy success [72]. As we can consider the immune interactions and their chronology at the maternal foetal interface with more accuracy, new concepts of local homeostasis implying regulatory T cells [73] [74], dendritic cells [75] or Th17 system [76] [77] are emerging. Environmental influences seem also fundamental on these local immune regulations [78] [79].

Given the revealed differences between classic immunology principles and the mechanisms controlling embryo implantation, we cannot consider anymore this phenomenon as a maternal tolerance to a foetal allograft [80]. Today, we describe a successful implantation and pregnancy as a dynamic and bidirectional dialogue between mother and conceptus, where the receptive endometrium seems to be a selective biosensor [21] towards a competent embryo [43] [44].

1.2.3. Key immune cells in reproductive immunology

Local immune interactions taking place at the maternal foetal interface are enabled by the presence of specific immune cell groups (Table 1) including uterine natural killer cells (uNK), macrophages, dendritic cells (DC) and T regulatory cells (Treg), which invade the endometrium during middle luteal phase via cyclic expression of local chemokines. Chemokines, such as CCL4 (Chemokine Ligand 4) also designated as Macrophage Inflammatory Protein-1 β (MIP-1 β), enable the recruitment and retention of specific immune cells in endometrium [81] [82].

During implantation window, uterine Natural Killers (uNK) form the majority of immune cell population in a receptive endometrium (Figure 9). Their phenotype and function differ from circulating Natural Killer (NK) cells called after their ability to immediate cellular lysis. The

presence of various families of receptors on NK cells helps to understand and predict their functions [83]. When considering their phenotypes, in majority, uterine NK cells are CD56(+) bright and CD16(-) dim whereas peripheral NK cells are CD56(-) dim and CD16(+) bright [84]. CD16 is a membrane protein involved in cellular lysis: CD16(+) NK cells are cytotoxic while CD16(-) NK cells secrete cytokines . If comparing CD56(+) NK cells from peripheral blood and endometrium, their repertoire of activating and inhibiting receptors are significantly different, resulting in distinct regulation of NK related activity. Uterine NK cells main function consists in cytokine production, but they keep their cytotoxic potentiality which could be triggered in an excessive inflammatory environment [85]. Cytokinic secretions of uNK cells are orientated towards angiogenesis and local vascular remodelling [86] allowing a sufficient trophoblastic invasion. When facing trophoblastic cells, particularly via the incoming HLA-C contact, uNK cells will fulfil their maturation and become decidual NK cells (dNK), a specific subset with immune modulatory potential which will direct a further placentation [87] [88].

Figure 9: Endometrial uNK immunostaining (CD56+) during middle luteal phase showing a normal invasion in a fertile control patient (b), an insufficient invasion (a) and an excessive invasion (c) in patients with repeated embryo implantation failure.

Endometrial slides from frozen samples, anti-CD56 Ventana antibodies



Macrophages are the second most abundant immune cell population in the secretory endometrial stroma [89]. Their presence is under cyclic hormonal control [90]: estradiol and

progesterone are shown to influence this endometrial influx [91]. Physiologically, macrophages in the endometrium are described as having a tolerogenic phenotype and mostly induce anti-inflammatory cytokine secretions [92]. They are also present at the further maternal foetal interface, contributing to foetal tolerance, trophoblast invasion, vascular remodelling and cellular migration [93]. Their excessive presence in decidua or a Th-1 dominant environment has been linked to preeclampsia and recurrent miscarriages [94].

Recently, a major interest was granted to an endometrial immune cell population interacting with uNK cells: the uterine Dendritic Cells (uDC). Dendritic cells belong to antigen presenting cells family and, besides pregnancy, they are enrolled in promoting immune responses and preventing autoimmunity. They are localised at maternal foetal interface during early pregnancy, where local signals drive them towards tolerogenic or immunogenic potential. In physiological circumstances, in addition to the promotion of an endometrial immune modulation, they are supposed to promote local angiogenesis, via their interaction with uNK cells, down regulating their activation markers and inducing their cytokinic production [95] [75].

A specific subgroup of T lymphocytes has also been identified as intervening at maternal foetal interface [96] [97]. T regulatory lymphocytes (Treg), powerful inhibitors of cell mediated immunity, seem to have a central role in preventing immunity against paternal antigens [98]. Sequential intervention of a series of chemokines and cytokines is suggested to promote Treg endometrial attraction. Apart from cyclic expansion under oestrogen and progesterone influence [82] [99], Treg cells are attracted in the endometrium by chemokines such as CCL3, CCL4 and CCL5 [100]. Their proliferation and maturation are further amplified by seminal plasma exposure through Transforming Growth Factor β (TGF β) and prostaglandins [101]. Suppressive functions and recruitment of Treg are then supposed to be optimised via antigen presentation [102], reducing the immune attacks towards the conceptus.

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Endometrial Immune Cell Type	uterine Natural Killer	Macrophage	Dendritic Cell	Regulatory T Cell
% of Endometrial Leukocytes	70 %	20 %	1-2 %	1-2 %
Major Secreted Cytokines	IL-10 IL-18 GM-CSF IFNγ VEGF LIF	IDO IL-10 GM-CSF G-CSF IL-12 VEGF PDGF	IDO IL-10 IL-6 TGFβ VEGF IL-15 IL-12	IL-10 TGFβ M-CSF LIF IL-35

IL: Interleukin, GM-CSF: Granulocyte-Macrophage Colony Stimulating Factor, IFN: Interferon, VEGF: Vascular Endothelial Growth Factor, LIF: Leukaemia Inhibitory Factor, IDO: Indoleamine 2,3-dioxygenase, G-CSF: Granulocyte-Colony Stimulating Factor, PDGF: Platelet-Derived Growth Factor, TGF: Transforming Growth Factor, M-CSF: Macrophage-Colony Stimulating Factor

1.2.4. Key cytokines in reproductive immunology

At the maternal foetal interface, the establishment of functional networks between different immune actors are mediated by small, soluble, labile and inducible glycoproteins called cytokines. Already in late 1980's, Wegmann described "the embryo bathing in a sea of cytokines" [103] and as underlined earlier in this chapter, given the extreme complexity of cytokines networks [60], only some selected ones which seem essential to embryo implantation are presented.

A major group of cytokines involved in reproduction are members of GP130 (Glycoprotein 130) family, amongst which Leukaemia Inhibitory Factor (LIF), Interleukin 11 (IL-11) and Interleukin 6 (IL-6). LIF is expressed in endometrium trough menstrual cycle with an increase in secretory phase and early pregnancy [104]. It stimulates cell proliferation, differentiation and survival [105] which are necessary to embryo development [106]. Its importance was

emphasized with LIF knockout murine models, where embryo implantation failed, whereas LIF knockout embryos did implant in a LIF producing foster mother [71]. Also, patients with primary unexplained infertility showed altered endometrial LIF secretion [107]. Interleukin 11 (IL-11) is also detected at the maternal foetal interface with cyclic variation and a higher expression during late secretory phase [108]. It's involved in endometrial decidualisation and local Natural Killer cells maturation [109] and further placentation [110]. Fertility is also impaired in IL-11 lacking murine models given an insufficient post implantation endometrial response [111]. Part of the GP130 family, interleukin 6 (IL-6) is a multifunctional cytokine, involved in immediate immune response and haematopoiesis. Its endometrial expression increases during middle luteal phase and its concentration is higher in decidual than in placental tissues [112]. The major epithelial localisation points out the IL-6 role in embryo attachment. Reduced fertility with lower embryo implantation is reported in IL-6 deficient mice [113].

The vascular tripod involving the interleukins 12, 15 and 18 (IL-12, IL-15, IL-18) is a major component of the endometrial cytokine network, particularly through the regulation of uterine Natural Killer (uNK) cells and the local angiogenesis induction [26]. Endometrial production of IL-15 is involved in the recruitment [114] [115] and the maturation of uNKs towards immune-modulatory cytokine producing cells [116] [117]. IL-15 knocked out mice show a lack of endometrial uNK cells and undecidualised spiral arteries [115]. IL-18 is a bivalent cytokine, also produced in the maternal foetal interface. When acting individually, it enhances uNK cells angiogenic cytokine production [118], promoting the local vascular remodelling. But when co-stimulated with IL-12, IL-18 drives uNK cells towards cytotoxicity and enhances local production of pro-inflammatory cytokines [119]. Moreover, some local immune modulators have been recently described for IL-15 and IL-18, which should be considered when evaluating these cytokines effects [120] [121]. During implantation window, variations in the endometrial expression of these vascular cytokines have been described in patients with unexplained reproductive failure when compared to fertile women [122] [123]. They therefore have been suggested as pre conceptual biomarkers to evaluate the immune local profile at the time of endometrial receptivity [24].

The interleukin 1 (IL-1) system is a considerable inflammatory mediator at the maternal foetal interface. This system includes two ligands, two membrane receptors, a non-binding

receptor accessory protein, and an antagonist receptor [124]. The components of this multifunctional inflammatory system [125] have been localised in the Human endometrium during the window of implantation, as well as in the placental tissues [36], and suggested to control the trophoblastic invasion [126].

On the other hand, the Transforming Growth Factor Beta (TGF- β) is a key local immune modulator essential to the embryo implantation process [127]. The TGF- β family, including three isoforms (TGF- β 1, 2, 3), has pleiotropic effects on cellular growth, differentiation and immune modulation. TGF- β is expressed in endometrial and decidual tissues [126]. TGF- β is involved in embryo implantation by helping embryo attachment, enabling local immune modulation, and controlling trophoblast invasion through regulation of locally secreted factors such as other growth factors, angiogenic factors, lytic enzymes like metalloproteases or other major pro implantation cytokines like LIF [128] [129] [130].

Other endometrial growth factors seem crucial for the local cell proliferation and differentiation leading to a successful embryo implantation. The Vascular Endothelial Growth Factor (VEGF) is one of the major factors involved in local angiogenesis and vascular remodelling essential to the establishment of a functional hemochorial placentation. Through a series of isoforms and activating or inhibiting receptors, the VEGF family enhances endometrial vascularization and vascular permeability [131]. Their action is under cyclic steroid control [132] [133] as well as locally secreted cytokines [134] and hypoxic conditions [135]. The Epidermal Growth Factor (EGF) is another local growth factor involved in trophoblastic invasion, proliferation and differentiation [136] [137]. EGF is localised in endometrial stroma, decidua and trophoblast [138]. Murine models lacking EGF or its receptors are shown to have impaired infertility, with early embryonic death, abortion or intrauterine growth restriction [139] [140]. Heparin Binding EGF Like Growth Factor (HB-EGF), which also shares receptors with EGF and TGF, is predominantly expressed during the window of implantation. It is localised in endometrial stromal and epithelial cells, regulating the local cell proliferation, secretion and decidualisation [141]. It is also described as a major mediator of embryo implantation by enabling the blastocyst attachment through membrane receptors on trophectoderm [142] [143].

Finally, Colony Stimulating Factors are major cytokines involved in reproduction. Their localisation in the reproductive tract and their immunotrophic, anti-apoptotic and immunomodulatory properties will be detailed in this work through dedicated articles.

1.3. Hypothesis and Objectives

In the present work, in order to help the understanding of Colony Stimulating Factors (CSFs) and particularly Granulocyte-Colony Stimulating Factor (G-CSF) contribution in reproductive field, we propose a way through three original articles. Our hypothesis was that G-CSF could modulate, by acting on some target genes, the pre-implantation endometrium which is ultimately described as a biosensor towards the embryo.

A first review article is dedicated to CSFs family, each member's localisation in reproductive tract, biological functions in reproduction and actual medical applications. The two other articles focus on G-CSF, already described as one of the most promising innovative therapies in reproductive medicine. By studying G-CSF and especially by detailing its endometrial actions, in women and murine models, we aim to illustrate how this cytokine could modulate the embryo implantation process.

G-CSF action on Human endometrium is studied in the second article. Based on a former endometrial transcriptomic study, we identified hypothetical targets of G-CSF. Then we described variations of expression for these targets in endometrium of infertile and control women. We finally tested G-CSF action on the specific targets, using a dynamic endometrial microhistoculture model.

The third article is about G-CSF supplementation on murine models. We chose specific crossings where the endometrial biosensor plays a major role between fertile and abortion prone models. The timing of the systemic G-CSF supplementation on females was also chosen in order to target specific endometrial action, avoiding complementary effect on ovulation or embryonic growth. We then studied, in both fertile and abortion prone models, G-CSF systemic supplementation effects on embryo implantation and early embryonic loss and compared it to control mice receiving placebo and to control non-injected mice.

2. Colony Stimulating Factors in Reproduction

2.1. Colony Stimulating Factors (CSFs)

2.2. CSFs Localisation in the Reproductive Tract

2.3. CSFs Functions in Reproduction

2.4. CSFs Medical Applications

2.5. Dedicated Original Article

2. Colony Stimulating Factors in Reproduction

2.1. Colony Stimulating Factors (CSFs)

The CSF (Colony Stimulating Factor) family includes: CSF-1 or M-CSF (Macrophage-Colony Stimulating Factor), CSF-2 or GM-CSF (Granulocyte-Macrophage-Colony Stimulating Factor), and CSF-3 or G-CSF (Granulocyte-Colony Stimulating Factor). These cytokines are studied from the mid 1960's and were named after their action on proliferation and differentiation of leukocytes (Figure 10).

Figure 10: General model of haematopoiesis, with CSFs localisation From Kaushansky, NEJM, 2006



HSC: hematopoietic stem cell CLP: common lymphoid progenitor CMP: common myeloid progenitor TNKs: T cells and natural killer cells GMs: granulocytes and macrophages MEPs: megakaryocytes and erythroid cells BCPs: progenitors for B cells NKPs : progenitors for NK cells TCPs : progenitors for T cells GPs : progenitors for granulocytes MPs : progenitors for monocytes EPs : progenitors for erythrocytes MkPs : progenitors for megakaryocytes IL: Interleukin SCF: stem-cell factor TPO: thrombopoletin EPO: erythropoletin CSF: colony-stimulating factor GM-CSF: granulocyte-macrophage CSF G-CSF: granulacyte CSF M-CSF: macrophage CSF

CSFs are 18-70 kDa labile glycoproteins, act through specific cell-surface receptors. Each of these transmembrane proteins includes one or two extracellular cytokine-binding domains containing approximately 200 amino acids, a transmembrane domain of 20 to 25 residues, and an intracellular domain of approximately 100 to 500 amino acids with the box 1 and box 2 motifs that recruit kinases of the Janus kinase (JAK) family.

Each of these cytokines supports the survival and proliferation of a number of distinct target cells via JAK-STAT signalling in an endocrine, paracrine or autocrine mode. The binding of the cytokine to its specific receptor induces a major conformational shift, bringing the two tethered cytoplasmic JAKs into close juxtaposition, thereby triggering activation of the kinases by mutual cross-phosphorylation. Once the JAKs are activated, a number of secondary signalling molecules are phosphorylated, each of which activates an overlapping subgroup of tertiary signalling molecules such as transcription factors, cell-cycle activators and inhibitors anti-apoptosis molecules and other growth factors which will be ultimately responsible for the specific effects induced.

Apart from inducing these cascades, the binding of the CSF to its receptor instigates internalization of receptors, activation of phosphatases, and production of suppressors of cytokine signalling, in order to terminate the stimulating signal.

Their involvement in reproduction was raised from early 1970's when a CSF activity was identified in the Human and murine placenta.

2.2. CSFs Localisation in the Reproductive Tract

All CSFs and their corresponding receptors are localised along the female reproductive tract, but each member is predominantly expressed in some specific locations. At the maternal foetal interface, GM-CSF and its receptor are the most studied. In the ovarian granulosa and follicular fluid, G-CSF seems to raise more interest in fundamental research and medical applications. In the seminal plasma, GM-CSF again is the most studied amongst the CSFs, particularly due to its action on pre implantation endometrium.

2.3. CSFs Functions in Reproduction

M-CSF and mostly G-CSF are described as involved in the ovulatory process. In embryo implantation, placental growth and embryo development, GM-CSF has the major implication, through its immune modulatory, angiogenic and trophic local actions.

2.4. CSFs Medical Applications

CSFs could be used as biomarkers. Serum variations of G-CSF and M-CSF during ovulation and hormonal ovarian stimulation suggest a possible use of the serum CSFs levels as predictive biomarkers in IVF outcome. Higher CSF serum levels have also been described in various reproductive pathologies such as preeclampsia, spontaneous preterm birth or repeated miscarriages. But most of all, the follicular G-CSF quantification has been presented as a pre-conceptual biomarker for the oocyte implantation potential and is in development for clinical application.

Given its major trophic actions, GM-CSF supplementation in embryo culture media during assisted reproductive therapies is under evaluation. Recent studies suggest a higher rate of ongoing pregnancies with its use, particularly in patients with history of recurrent miscarriage.

Finally, G-CSF supplementation seems to be one of the most innovative therapies in reproductive medicine. Some attempts of G-CSF systemic supplementation have already been reported in case of dysovulation. Intra uterine instillation of G-CSF has been suggested to solve cases of endometrial trophic defect unresponsive to usual therapeutics. But most of all, G-CSF systemic supplementation is currently evaluated to improve uterine receptivity, in patients with history of repeated miscarriages or embryo implantation failure.

2.5. Dedicated Original Article

The following article is a review dedicated to CSFs in reproduction. It was submitted to Journal of Reproductive Immunology.

"Review Article"



From Sempe, Le Petit Nicolas, 1960

Colony Stimulating Factors 1, 2, 3 and Early Pregnancy Steps:

From Bench to Bedside

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Abstract

Reproductive immunology applies general immunology principles to specialised targets, reproduction and development. Involvement of Colony Stimulating Factors (CSFs) in reproduction illustrates this. The CSF family includes CSF-1 or M-CSF (Macrophage-CSF), CSF-2 or GM-CSF (Granulocyte Macrophage-CSF), and CSF-3 or G-CSF (Granulocyte-CSF). Each member has a specific localisation and timed-expression in the reproductive tract with specific functions involving them in ovulation, embryo implantation, placentation and further embryonic development. They are used in reproductive medicine either as biomarkers of oocyte quality and competence (follicular G-CSF), or to supplement embryo culture media with human recombinant GM-CSF or are used as an innovative therapy by using human recombinant G-CSF for infertile patients. Given fundamental considerations on CSFs and their strong implication in reproduction, this review aimed to detail the current knowledge for each member of the family to improve our understanding on their implication in the maternal foetal cytokinic dialogue and possibly preventing reproductive disorders.

Key Words

Colony Stimulating Factors, Early Pregnancy, Embryo Implantation, Reproductive Tract

1. Introduction

The CSF (Colony Stimulating Factor) family includes: CSF-1 or M-CSF (Macrophage-Colony Stimulating Factor), CSF-2 or GM-CSF (Granulocyte-Macrophage-Colony Stimulating Factor), and CSF-3 or G-CSF (Granulocyte-Colony Stimulating Factor). CSFs are 18-70 kDa labile glycoproteins, act through specific membrane receptors, via JAK-STAT signalling pathways in an endocrine, paracrine or autocrine model [1].

These cytokines are studied from the mid 1960's and were named after their action on proliferation and differentiation of leukocytes. Their involvement in reproduction was raised from early 1970's, when it was demonstrated, in mouse and Human, that placental media could stimulate hematopoietic cell multiplication [2]. Then higher levels of CSFs were described in murine pregnant uterus. CSF-2/GM-CSF was then demonstrated by Athanassakis and Wegmann to be a growth factor for the placenta whereas a key role for CSF-1/M-CSF was in the same period demonstrated by Pollard's group. Messenger expression and protein production for these three cytokines and their receptors were further identified in the reproductive tract, especially in the ovary and at the maternal foetal interface. CSFs concentration variations, under different reproductive conditions, are also described in serum and follicular fluid, suggesting the use of CSFs as possible predictive biomarkers in reproductive medicine.

We will detail in this review what is now admitted in reproduction for each member and will describe the experiments which allowed highlighting their immuno-trophic, anti-apoptotic and immuno-modulatory actions during the early steps of pregnancy.

During the past five years, these cytokines took a large part in innovative therapies in reproductive medicine, particularly for two of them: CSF-2/GM-CSF and CSF-3/G-CSF. We will review their utilisation as biomarkers, their supplementation in embryo culture media to enhance the embryo quality, or their supplementation in different cases of reproductive failure such as dysovulation, repeated pregnancy loss and embryo implantation failure.

2. CSFs localisation in the reproductive tract

2.1. Materno-foetal interface

A Colony Stimulating Factor activity was traced as early as 1980 in Human placenta conditioned medium and in the 90's for the Human placenta, decidua and endometrium [3].

At the materno-fetal interface, the presence of CSF-1/M-CSF was assessed quantitatively and, while serum levels were enhanced two fold during pregnancy, the uterine concentration was enhanced 1000 fold, strongly suggesting an important role for gestation [4]. This observation was completed in mice by the localisation and temporal expression of the cytokine in endometrium and decidua, coupled with the placental trophoblast expression of its receptor [5] [3].

At the same time, a direct trophic role was demonstrated for CSF-2/GM-CSF and raised the concept of immunotrophism [6]. The proof of immunotrophism came with the further demonstration that activated T cells secreted soluble factors, including CSF-2/GM-CSF, which acted as growth factors for the placenta [7]. Likely due to its effects in murine abortive models [8] [9], CSF-2/GM-CSF has been the most studied CSF family member in the materno-foetal interface. CSF-2/GM-CSF and its receptor productions were first localised by Robertson and Kanzaki in murine trophoblast and reproductive tract [10]. CSF-2/GM-CSF secretion by uterine epithelial cells was interestingly demonstrated to be maintained in a variety of lymphocyte deficient mice, establishing the secretion of an immune mediator by non-immune cells in the reproductive tract. CSF-2/GM-CSF and its receptor production were also confirmed in Human female reproductive tract [11]. The membrane receptor was identified on endometrial immune cells such as macrophages, granulocytes and dendritic cells [10]. Endometrial CSF-2/GM-CSF production was shown to be regulated by oestrogen, progesterone and TGF beta 1.

Approximately in the same period, the presence of CSF-3/G-CSF and its receptors in the reproductive tract was also established: in the placenta and the decidua [12]. In mice, the cytokine and its receptor are shown to be expressed spongiotrophoblasts and placental labyrinths, as well as in decidua basalis and endometrial epithelium. Likewise, they were

detected in Human cytotrophoblasts and syncytiotrophoblats on placental side, and also in maternal side, in decidual stromal cells, endometrial glands and epithelium and local Natural Killer cells.

2.2. Ovary: granulosa and follicular fluid

Ovarian CSFs are mostly studied in Human. Each CSF and its receptor are localised in the granulosa at protein and mRNA level. Their concentration is higher in the follicular fluid than in serum. Among all of them, follicular CSF-3/G-CSF seems to raise more interest in fundamental research and medical applications [13] [14].

2.3. Seminal plasma

All three CSFs are detected in Human seminal fluid at low concentration, but their interest appears higher in mammalian study models without cervical barrier such as mouse or pig, where CSF-3/G-CSF might modulate local cell mediated immunity and TGF beta 1 appears as a major regulator of endometrial CSF-2/GM-CSF [15].

3. CSFs functions in reproduction

3.1. Ovulation

In mice, Pollard's team took advantage of the total absence of CSF-1/M-CSF described in a macrophage-deficient osteopetrotic mouse model (op-/-) and demonstrated that pregnancy was impaired in such females [16]. Later on, they showed that females had extended oestrus cycles and poor ovulation rates. Interestingly, systemic administration of CSF-1/M-CSF restored a variety of defects due to the op-/- mutation but did not restore normal fertility, proving the importance of the other CSF members' synthesis. Studies using a CSF-1/M-CSF transgene showed that transgene expression indeed corrected all the reproductive defects, besides allowing a more precise evaluation of the site of production [17]. In other species, presence of CSFs in the ovary at the time of ovulation has been traced: in rodents, cow, buffalo, horse, dog and hen.

In Human, CSF-1/M-CSF, CSF-3/G-CSF, and limited CSF-2/GM-CSF were all found first in supernatants of cultured ovarian epithelium cells [18].

Later on, cyclic serum changes including a peri-ovulatory peak, higher serum concentration after ovarian hyper-stimulation [14] and higher follicular concentrations [13] suggest a major role for CSF-3/G-CSF in the ovulation process. CSF-3/G-CSF has been suggested to be implicated in the local follicular inflammation [19], via leukocyte attraction and activation, leading to ovulation [20]. However, in mice model lacking only CSF-3/G-CSF, ovulation impairment is not described [21]. Interestingly, in a murine model of ovarian transplantation recently described [22], CSF-3/G-CSF supplementation seems to improve the viability and function of the graft by maintaining primordial follicles.

Similarly, CSF-1/M-CSF serum levels were found elevated, after ovarian hyper-stimulation for IVF, in patients who became pregnant. These higher serum levels were suggested to be an additional predictive parameter of the IVF outcome [23]. The origin could be the cumulus oophorus cells, since CSF-1/M-CSF has been traced there, and its production shown to be highly progesterone dependent.

Finally, CSF-2/GM-CSF has also been traced in the follicular fluid of patients undergoing ovarian stimulation for ICSI [24]. In rodents, Tamura et al suggested that the origin of this follicular CSF-2/GM-CSF was local immune cells.

3.2. Embryo implantation

At the maternal foetal interface, CSFs are an integral part of the utero placental cytokine network, needed to establish and maintain pregnancy. They are suggested to contribute together to create a dominant Th-2 environment, suitable for the establishment and maintenance of a local tolerant immune environment required for a successful implantation.

CSF membrane receptors are identified on local immune cells and immuno-modulatory actions are described for each CSF.

Outside pregnancy, after a hematopoietic allogenic transplantation, systemic CSF-3/G-CSF induced Th-2 environment is shown to reduce Graft-Versus-Host disease. This Th-2 switch is an example of CSF-3/G-CSF tolerance induction properties, via IL-10 or IL-4 production and IL-2, IFN γ or TNF α inhibition. IL-10 pathway seems to be a major factor in these immune modulatory systemic effects: its production is enhanced either by CSF-3/G-CSF mobilized cells [25] or regulatory T-cells [26].

Local uterine production of CSF-3/G-CSF may contribute to the modulation of uterine Natural Killers' cytotoxicity, reducing for example IFNy and IL-18 production [27].

CSF-1/M-CSF also participates to this tolerogenic environment by maintaining homeostasis between local macrophages and dendritic cells and shaping them with regulatory properties [28].

But most of all, CSF-2/GM-CSF appears as an essential local modulator implicated in embryo implantation [29]. It was shown that the murine decidua of aborting CBA x DBA/2 mating combination contained less GM-CSF than those of non-aborting mating murine combinations, such as CBA x BALB/c. This was followed by the first correction of murine abortion in the CBA x DBA/2 system by CSF-2/GM-CSF injection [9]. Later on, it was shown

that CSF-2/GM-CSF was able to control uterine cell cytotoxicity [30]: neutralising CSF-2/GM-CSF would increase spontaneous abortion while supplementing CSF-2/GM-CSF would enhance fertility.

Recently, CSF-2/GM-CSF production has been also attributed to Natural Killer cells (NK), and not solely to T cells. Interestingly, maternal uterine NK (uNK) cell-activating receptor KIR2DS1 enhances placentation by inducing decidual NK (dNK) cell production of CSF-2/GM-CSF. This CSF-2/GM-CSF has a direct effect on migration of primary trophoblast cells and JEG-3 choriocarcinoma cells in vitro [31].

Moreover, at endometrial level, CSF-2/GM-CSF membrane receptors are identified on the major uterine immune cells during implantation period [10]. Latest experiments, using GM-CSF lacking mice, also point how this cytokine is responsible for setting T cell tolerance via dendritic cell regulation during early pregnancy [32].

Apart from local immune modulation, CSFs might have an angiogenic action. This action is already described in clinical trials on coronary revascularization with CSF-2/GM-CSF and CSF-3/G-CSF supplementation, or on vascular repair in diabetes. This potential may be due to induced increase of endothelial progenitor cells and pro-angiogenic gene expression in monocytes [33].

3.3. Placental growth

All CSFs do enhance placental growth and differentiation. However the main trophic role is attributed to CSF-2/GM-CSF.

This placental trophic role was first demonstrated in mice [7] [34]. In vitro, CSF-2/GM-CSF promotes placental growth [35]. Its neutralisation by antibodies inhibits trophoblastic proliferation [36]. It also stimulates cytotrophoblastic differentiation [36]. In vivo, proof of trophic action is obtained by differential weights of placenta when comparing normal to abortion prone, nude or CD4 lacking mice [9] [37] [38]. In CSF-2/GM-CSF deficient mice [39], fertility impairment consequences linked to placental architectural and functional

abnormalities is reported, including higher embryonic resorption rate and lower foetal weight.

In Human, the CSF-2/GM-CSF role on placental cell growth in vitro was first demonstrated by Loke [40] using precisely the invasive extravillous trophoblast subset. CSF-2/GM-CSF is also recently described as a placentation enhancer by improving trophoblast migration as a result of its production by decidual Natural Killer cells via their KIR2DS1 interaction [31].

Finally, in ruminants, CSF-2/GM-CSF was shown to enhance the production of a major trophoblast promoter, interferon tau [41].

3.4. Embryo development

CSF-2/GM-CSF implication in embryo development and foetal survival was first described in mice [7] [8]. At embryonic level, murine blastomere viability is enhanced by CSF-2/GM-CSF [42].

It also promotes embryo development in pig, sheep and cow, particularly by increasing IFN tau secretion in the latter two [41].

The positive trophic CSF-2/GM-CSF effects on Human embryos are studied from the late 1990's [43] [44]. Cellular mechanism implicated in this embryonic effect, by supplementing culture media with Human recombinant GM-CSF, is blastomere protection against apoptosis [45] without apparent effect on embryonic chromosomal constitution [46] [47].

4. CSFs medical applications: From bench to bedside

4.1. Using CSFs as biomarkers

At the fundamental level, cyclic, post hormonal stimulation, and inter-individual variations of serum concentration for CSFs are shown. These basic data suggest a possible use of the serum CSFs levels as predictive biomarkers in IVF outcome [48] [23] for CSF-3/G-CSF and CSF-1/M-CSF.

Different teams also suggested this use in various reproductive pathologies such as preeclampsia, spontaneous preterm birth or repeated miscarriages [49]. Globally, higher levels of serum CSF seems linked to these pathologies. But these data were collected only on few unrepeated studies, involving small cohorts of patients.

Moreover, CSF-3/G-CSF concentration differences in individual follicular fluids were correlated to the potential of the corresponding oocytes to give a subsequent birth after fertilization, in hyper-stimulated or natural IVF cycle. The follicular CSF-3/G-CSF quantification has therefore been patented as a pre-conceptual biomarker for the oocyte implantation potential [50] [51] [52] and is in development for clinical application (Diafert[™]).

4.2. Embryo culture media supplementation

Essential data on embryo-trophic effects of CSF-2/GM-CSF are discussed since nearly 25 years and recent studies about its mechanisms of action on embryo development led to the elaboration of new embryo culture media supplemented with Human recombinant CSF-2/GM-CSF (EmbryoGen®). A recent randomized study suggests a higher rate of ongoing pregnancies with use of CSF-2/GM-CSF in embryo culture media (EmbryoGen®), particularly in patients with history of recurrent miscarriages [53]. A prospective placebo-controlled and double-blinded study was conducted to demonstrate that CSF-2/GM-CSF supplemented media had no effect on embryonic chromosomal constitution [46]. However, epigenetic effects cannot be totally excluded. It should also briefly be recalled that CSF-2/GM-CSF,

through a cellular feeder layer, was one of the theoretical basis for Human embryo culture on Vero cells or Human decidual cells (Endocell[®]).

Low serum CSF-2/GM-CSF concentrations observed in women with recurrent miscarriages has also been claimed to be corrected by intravenous immunoglobulin treatment [49]. It is also interesting to note that correction of CSF-2/GM-CSF levels has been suggested to be the explanation for success of some herbal medicine treatments. More classically, a correction of CSF concentrations has also been reported in cases of progesterone treatments [54].

4.3. Innovative therapies

Human recombinant CSF-3/G-CSF (Filgrastim, Neupogen[®]) is used by subcutaneous administration since late 1980's in haematological indications such as chemotherapy induced neutropenia, congenital agranulocytosis or haematopoietic stem cell transplantation.

In reproductive medicine, new applications of Human recombinant CSF-3/G-CSF are recently described

CSF-3/G-CSF have been suggested to be involved in polycystic ovarian syndrome [55]. Few attempts of CSF-3/G-CSF use in case of dysovulation have been reported [20]. Beside ovulation related problems, another emerging field of application related to CSF-3/G-CSF is to improve the uterine receptivity.

A randomised study using CSF-3/G-CSF supplementation on IVF stimulation protocols, in case of repeated miscarriages, suggests a higher birth rate and fewer cases of pregnancy loss [56] [57]. Moreover, CSF-3/G-CSF supplementation has been tested in preliminary IVF protocols involving patients with history of embryo implantation failure [58].

There are also cases of pregnancy reported after intra-uterine instillation of CSF-3/G-CSF, in patients with endometrial trophic defect and history of IVF failure [59] [55] [60].

5. Conclusion

CSF family is an illustration of the scope of reproductive immunology. These immune cytokines and their receptors are localised along the reproductive tract, and not solely in immune cells. They are under cyclic hormonal regulation.

Through various effects on local inflammation, immuno-modulation or immuno-trophicity, they act on reproductive function at different levels: ovulation, embryo implantation, placentation and embryo development.

Fundamental studies on CSFs, their localisation, their physiological or pathological variations, their various actions, have generated direct applications in reproductive medicine.

Human recombinant CSF-2/GM-CSF supplementation is tested in embryo culture media. Follicular CSF-3/G-CSF is suggested as a pre-conceptual non-invasive biomarker of oocyte competence in IVF. Locally or systemically administrated Human recombinant CSF-3/G-CSF is used in various medical trials involving infertile patients with dysovulation, unexplained repeated miscarriages or embryo implantation failures.

Despite their undeniable interest in reproductive field, CSFs actions are still not elucidated, especially concerning long term effects of CSF used on early stages of embryo development. Doses and timing of CSF administration in case of infertility also remain unestablished. The pro-inflammatory or immuno-modulatory effect of CSFs seems to depend on the dose. A wrong timing induced in the pre-implantation process might interfere with a local convenient immune environment and turn prejudicial for embryo implantation and development.

This is a question of major interest, knowing that a succession of inflammatory reaction and tolerogenic environment is crucial for a successful embryo implantation.

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3. G-CSF Effects on Human Endometrium

3.1. G-CSF in Reproduction

3.2. Contribution of a Previous Large Scale Microarray Study

3.3. Use of a Previously Described Endometrial Ex Vivo Model

3.4. Dedicated Original Article

3. G-CSF Effects on Human Endometrium

3.1. G-CSF in Reproduction

G-CSF supplementation appears today as one the most promising therapies in reproductive medicine. This innovative therapy is proposed in various schemes of supplementation, either systemic, by subcutaneous administration, or local, by intra uterine infusion. The medical indications are not strictly defined either: G-CSF is reported to be used in different cases of reproductive failure such as unexplained recurrent miscarriages, repeated embryo implantation failures or endometrial trophic defect.

G-CSF and its receptor have been localised in the female reproductive tract. GCSF's inflammatory, immune modulatory or trophic effects have been evoked in different models, but the endometrial mechanisms of action by which G-CSF would positively influence the embryo implantation are largely unknown. The objective of the present study was to identify the possible relevant pathways influenced by G-CSF, involving target genes preselected from a previous large scale microarray and using an ex-vivo model of endometrial microhistoculture.

3.2. Contribution of a Previous Large Scale Microarray Study

Hypothetical pathways and molecular interactions putatively regulated by G-CSF were selected on the basis of gene expression deregulations according to the indications stemming from a previous large scale endometrial microarray study. This microarray was realised by comparing endometrial gene expressions during middle luteal phase, between fertile women, patients with a history of unexplained embryo implantation failure and patients with idiopathic repeated miscarriages. This microarray analysis suggested pre conceptual extensive endometrial deregulations. When analysing those intricate pathways involving G-CSF, the expression of some gene seemed specifically deregulated. The previous article dedicated to this microarray study is available in annexes.

Selecting among these highly deregulated genes, those hypothetically depending on G-CSF action, we chose those involved in immune regulation, coagulation system or integrins. We therefore selected the following genes, suggested as G-CSF targets in the endometrium: G-CSF Receptor (G-CSFR), Integrin alpha-V/beta-3 (ITGB3) known to be implicated in cell migration and embryo implantation, Plasminogen Activator Urokinase Receptor (PLAUR) described as interacting with integrins and implicated in cell migration, Thymidine Phosphorylase(TYMP) implicated in local angiogenesis, CD40 and CD40 Ligand (CD40L) involved in cell proliferation control.

We first confirmed the endometrial variations of these genes expressions in fertile women and patients with reproductive failure. Then we used an ex vivo model to illustrate G-CSF supplementation effects on endometrial expression of these genes.

3.3. Use of a Previously Described Endometrial Ex Vivo Model

The endometrial microhistoculture used in our study has been previously described as a functional ex vivo model. Endometrial samples taken during middle luteal phase were placed on collagen sponge gels, in a specific medium daily supplemented with estradiol and progesterone. In this model, the cellular functionality and differentiation are preserved for five days. The dedicated article to this model is presented in annexes.

On these ex vivo culture, we used a scheme of three consecutive day supplementation. For G-CSF stimulation, the five culture conditions were: G-CSF at either 20, 100 or 200 ng/ml, or G-CSF blocking antibody at 3 μ g/ml, and a control culture condition without G-CSF or antibody. To specifically target the G-CSF endometrial action, the same experiments were performed with Granulocyte-Macrophage-CSF (GM-CSF) at either 20, 100 or 200 ng/ml, or GM-CSF blocking antibody at 3 μ g/ml.

Recombinant protein or antibody was added to the culture medium every day during three consecutive days. After a three days incubation, the endometrial samples were collected in a RNA stabilizer solution for RNA extraction and RT-qPCR, for evaluation of the expression of all the considered endometrial target genes.

3.4. Dedicated Original Article

The details and the results of this study are described in a dedicated article entitled: "Granulocyte-Colony Stimulating Factor related pathways tested on an endometrial ex-vivo model", which was submitted to PlosOne and accepted.

"Research Article"



From Sempe, Le Petit Nicolas, 1960
Granulocyte-Colony Stimulating Factor related pathways tested on an endometrial ex-vivo model

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Abstract

Recombinant human Granulocyte-Colony Stimulating Factor (rhG-CSF) supplementation seems to be a promising innovative therapy in reproductive medicine, used in case of recurrent miscarriage, embryo implantation failure or thin endometrium, although its mechanisms of action remain unknown. Our aim was to identify possible endometrial pathways influenced by rhG-CSF.

Hypothetical molecular interactions regulated by G-CSF were designed through a previous large scale endometrial microarray study. The variation of endometrial expression of selected target genes was confirmed in control and infertile patients. G-CSF supplementation influence on these targets was tested on an endometrial ex-vivo culture. Middle luteal phase endometrial biopsies were cultured on collagen sponge with or without rhG-CSF supplementation during 3 consecutive days. Variations of endometrial mRNA expression for the selected targets were studied by RT-PCR.

At the highest dose of rhG-CSF stimulation, the mRNA expression of these selected target genes were significantly increased if compared with their expression without addition of rhG-CSF. The selected targets were G-CSF Receptor (G-CSFR), Integrin alpha-V/beta-3 (ITGB3) implicated in cell migration and embryo implantation, Plasminogen Activator Urokinase Receptor (PLAUR) described as interacting with integrins and implicated in cell migration, Thymidine Phosphorylase (TYMP) implicated in local angiogenesis, CD40 and its ligand CD40L involved in cell proliferation control.

RhG-CSF seems able to influence endometrial expressions crucial for implantation process involving endometrial vascular remodelling, local immune modulation and cellular adhesion pathways. These variations observed in an ex-vivo model should be tested in-vivo. The strict indications or counter indication of rhG-CSF supplementation in reproductive field are not yet established, while the safety of its administration in early pregnancy on early embryogenesis still needs to be demonstrated. Nevertheless, rhG-CSF appears as a promising therapy in some difficult and unsolved cases of reproductive failure. Indications of pre-conceptual rhG-CSF supplementation may derive from a diagnosed lack of endometrial expression of some target genes.

Key Words

G-CSF; GM-CSF; Embryo Implantation; Human Endometrium; Microhistoculture; TYMP; PLAUR; ITGB3; CD40; Recurrent Misscariages; Embryo Implantation Failure

Introduction

Recombinant Human Granulocyte-Colony Stimulating Factor (rhG-CSF) is used since late 1980's in haematological indications such as chemotherapy induced neutropenia [1], congenital agranulocytosis [2] or haematopoietic stem cell transplantation [3].

In reproductive medicine, rhG-CSF supplementation seems to be one of the most promising innovative therapies. Indeed, in distinct countries, rhG-CSF supplementation, either systemic (subcutaneous administration) or local (intra uterine infusion), is evaluated in the context of some unexplained recurrent miscarriages, repeated embryo implantation failures or thin unresponsive endometrium. Two randomised studies using rhG-CSF supplementation on IVF stimulation protocols, in case of repeated miscarriages, suggest a higher live birth rate and fewer cases of pregnancy loss [4] [5]. Moreover, rhG-CSF supplementation is tested in preliminary IVF protocols involving patients with a history of repeated embryo implantation failures (IF) [6]. There are also cases of pregnancy reported after intra-uterine infusion of rhG-CSF, in patients with IVF failure with endometrial trophic defect [7] [8] [9] [10].

However, the mechanisms of action by which rhG-CSF would positively influence the embryo implantation are largely unknown. The objective of the present study was to identify the possible relevant pathways influenced by a local administration of rhG-CSF, involving target genes pre-selected from a previous large scale microarray [11], using an exvivo model of endometrial microhistoculture [12]. In this model and considering the selected target genes, rhG-CSF seems able to influence some endometrial expressions crucial for the implantation process. These molecules whose expression seems to be under G-CSF influence are involved in the endometrial vascular remodelling, the local immune modulation and cellular adhesion systems.

Materials and Methods

Patients

81 women were enrolled for this study. All patients were involved in Assisted Reproductive Technology (ART) programs and were less than 38 years of age. There was no statistically significant difference in age and BMI between the different groups. All patients were nonsmoker. They all provided a written informed consent and this investigation was approved by our Institutional Review Board, Agence de la Biomedecine, under the trial registration number 2013-A00072-43.

17 patients were fertile control women, involved in ART because of male infertility. All had delivered after either intra-uterine insemination or ICSI within the two first attempts. They were evaluated at least six months after their delivery and before a new attempt. The mean age in this group was 33 years (from 29 to 35 years old). The mean Body Mass Index (BMI) was 23.8 (from 21 to 27.9).

19 patients had repeated unexplained embryo implantation failure (IF) [13] defined as an absence of pregnancy despite the transfer of 10 or more good quality embryos (fresh or frozen-thawed) over several cycles. All embryos had a fragmentation rate of less than 20% and were at least at the 4-cell stage by day 2 after IVF. The inclusion criteria also included a normal uterine morphology (excluding fibroma, polyps, thin endometrium, synechia, uterine malformation) after ultrasonography, hysterosalpingography and hysteroscopy, a normal karyotype, a normal hormonal reserve (FSH < 10 mIU/ml, antral follicle count > 9 follicles) and normal responses to the hormonal stimulation (with more than 8 oocytes retrieved per cycle). Normal endometrial trophicity was defined after ultrasonography by an endometrial thickness over 7 mm and an endometrial volume over 2.5 cm3 during luteal phase. In this group, the mean age was 31.7 years (from 28 to 35) and the mean BMI was 22.6 (from 19 to 29).

15 patients had a history of unexplained recurrent miscarriages (RM) [14] defined by at least three subsequent pregnancy losses between 6 to 12 weeks of gestation, remaining unexplained after uterine morphology evaluation (ultrasonography, hysterosalpingography, hysteroscopy), standard auto-immune exploration (antiphospholipid antibodies as lupus anticoagulant and anticardiolipin antibodies), evaluation of the thrombophilic activity (protein S, protein C, factor V, factor VIII) and exploration of genetic markers (parental karyotype). The mean age in RM group was 31 years (from 25 to 36) and the mean BMI was 20.6 (from 18 to 25).

30 other patients participated to our study. They were involved in a pre IVF cycle endometrial biopsy program (pre IVF "endometrial scratching" protocol). These endometrial biopsies were used for ex-vivo microhistocultures.

Endometrial Biopsies

Endometrial biopsies were programmed during a monitored natural cycle, 7 to 9 days after the ovulation surge (LH surge), during the hypothetical implantation window. These biopsies were performed with a standard Cornier pipelle (CCD Laboratories, Paris, France), collecting material from superficial endometrial layers in order to minimise sampling variations.

One part of the endometrial sample was formalin-fixed and paraffin-embedded for later histological study. We used a standard haematoxylin and eosin staining protocol on 3μ m thick sections for routine histological evaluation. Moreover, a histological endometrial dating, to classify patients as "in phase" or "out phase", was performed according to the Noyes criteria [15].

Another part was collected in RPMI 1640 Glutamax medium for endometrial ex-vivo culture.

A third and last part of the endometrial sample was transferred into an RNA stabilizer solution (RNA Later, Qiagen, Courtaboeuf, France) for further RNA extraction.

Endometrial Microhistoculture

The endometrial microhistoculture model used in this study has been previously described by our team [12]. In this ex-vivo model, the cellular functionality and differentiation are preserved for five days. As a brief reminder, endometrial samples (1 to 2mm3) were taken from biopsies collected in RPMI 1640 Glutamax medium, during the 2 or 3 first hours after collection. We put these endometrial blocks on collagen sponge gels (GelfoamH, Pharmacia Upjohn, Kalamazoo, MI, USA) which are placed into supplemented RPMI 1640 Glutamax culture medium. The medium supplementation was as follows: 15% heat-inactivated foetal calf serum, 1% penicillin and 1% streptomycin, non-essential amino acids, 1% sodium pyruvate (all from Gibco BRL, Life Technologies, France), 50 nmol/l of estradiol and progesterone (Sigma, St Quentin-Fallavier, France). Estradiol and progesterone supplementations were repeated daily. The cultures were maintained at 37° C in a 5% CO2 humid atmosphere.

For rhG-CSF stimulation, the five culture conditions were: rhG-CSF at either 20, 100 or 200 ng/ml, or G-CSF blocking antibody (anti G-CSF) at 3 µg/ml (all recombinant proteins and antibodies from R&D Systems, Lille, France). Recombinant protein or antibody was added to the culture medium every day during three consecutive days. After a three days incubation, the endometrial samples were collected in a RNA stabilizer solution (RNA Later, QIAGEN, Courtaboueuf, France) for RNA extraction and RT-qPCR, as described in the corresponding paragraph.

To specifically target the rhG-CSF endometrial action, the same experiments were performed with Granulocyte-Macrophage-CSF (rhGM-CSF) at either 20, 100 or 200 ng/ml, or GM-CSF blocking antibody (anti GM-CSF) at 3 μ g/ml (recombinant proteins and antibodies from R&D Systems, Lille, France).

The biopsies from 30 patients, involved in a pre IVF cycle endometrial biopsy program, were used for these ex-vivo microhistocultures. Biopsies from 17 patients provided 57 endometrial samples (1 to 2mm3) for rhG-CSF microhistocultures. Biopsies from 13 patients provided 39 samples (1 to 2mm3) for control rhGM-CSF microhistocultures.

Every patient was her own control, e.g. for each stimulation condition, an endometrial sample from the same patient was concomitantly placed in identical culture conditions, without the corresponding recombinant protein or blocking antibody. For these control cultures, only estradiol and progesterone were daily added (EP condition), as described, during the three days incubation period. Therefore, for the same endometrium, we could compare the target gene expressions under specific stimulation and EP condition.

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Endometrial RNA extraction, quality control and cDNA synthesis

The total endometrial sample was homogenized with an Ultra Turrax T15 (IKA-WERKE) and the homogenate was then purified on Qiashredder columns (Qiagen, Coutaboeuf, France).The total RNA was isolated with the Qiagen RNeasy mini kit (Qiagen, Courtabeuf, France) including the RNase-free DNAse set. Recombinant RNase inhibitor (10 units/µl of extracted RNA) was added to prevent RNA degradation. RNA quantity and quality were confirmed by the analysis with an Experion system and RNA Std Sens analysis kit (Bio-rad, Marnes la Coquette, France). The RNA was then stored at -80° C. The RNA (1 µg) was first reverse-transcribed into cDNA using random primers and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

Target genes selection and Real Time quantitative PCR (RT-qPCR)

Hypothetical pathways and molecular interactions putatively regulated by G-CSF were selected on the basis of gene expression deregulations according to the indications stemming from a previous large scale endometrial microarray study [11]. Briefly, this microarray analysis suggested pre conceptual extensive endometrial deregulations in patients with IF or RM. When analysing those intricate pathways involving G-CSF, the expression of some gene seemed strikingly specifically deregulated. Selecting among these highly deregulated genes, those hypothetically depending on G-CSF action, we picked up those molecules involved in immune regulation, coagulation system or integrins. We therefore selected the following genes, suggested as G-CSF targets in the endometrium: G-CSF Receptor (G-CSFR), Integrin alpha-V/beta-3 (ITGB3) known to be implicated in cell migration and embryo implantation, Plasminogen Activator Urokinase Receptor (PLAUR) described as interacting with integrins and implicated in cell migration, Thymidine Phosphorylase (TYMP) implicated in local angiogenesis, CD40 and CD40 Ligand (CD40L) involved in cell proliferation control.

Specific primers for these targets genes and Ribosomal Protein L13A (RPL13A), as the reference gene, were constructed using the Universal Probe Library Assay Design Center

(www.rocheapplied-science.com) and their sequences were searched against Gen-Bank sequences with the BLAST program to ensure the specificity of primers. Real-time PCR was carried out using a LightCycler 480 apparatus (Roche, Meylan, France). Reactions were set up using the following final concentrations: 0.5 mM of sense and antisens primers, 1X 480 SYBER Green master mix and 4 ml of 1/20 diluted cDNA. Cycling conditions were as follows: denaturation (95° C for 5 min), amplification and quantitation (95° C for 10 s, 60°C for 10 s and 72° C for 15 s) repeated 40 times, a melting curve program (65–95° C with a ramp rate of 2.2 °C /s) and a cooling step to 4° C. In addition to the no-reverse transcription and notemplate controls, an independently inter run calibrator (IRC) was included in each RT-PCR assay. This IRC was obtained from blast-cells. In each assay, an aliquot of the IRC cDNA was 20 times diluted and was submitted to the qPCR protocol as the unknown samples. PCR efficiencies for each quantified target and reference were calculated using known serial dilutions of each specific cDNA. Data were analysed using the LightCyclerH480 Software release 1.5.0. Each specific target transcription level was normalized to the geometric mean of the transcription levels of the reference gene, using the Advanced Relative Quantification of the LightCyclerH480 Software. Efficiency was controlled for each specific gene amplification.

For each sample, the results are expressed in concentration ratio (target gene mRNA level/reference gene mRNA level). For microhistoculture samples, these ratios are compared between the stimulated culture condition and the EP basal condition, as described in the dedicated paragraph, each patient being her own control.

Statistics

The Wilcoxon test was used to compare target genes mRNA expressions in each patient group and in each culture condition. The Spearman test was used to search for a correlation between different gene expressions. The statistical assessments were performed using the Stat View software (Abacus Concepts, CA, USA). The significance level was set up at p<0.05.

Results, Discussion, and Conclusions

Results and Discussion

Both the protein production and mRNA expression of G-CSF receptor (G-CSFR) have been shown to be localised at the maternal foetal interface since the late 1980's [16], with cyclic and gestational regulations [17] [18] and a trophoblast growth promoting role [19]. When considering the G-CSFR mRNA expression in control vs. infertile patients, we did not notice any significant global difference. But when considering the IF subgroup within the infertile patients, the G-CSFR mRNA expression was significantly lower (p = 0.01), as shown in figure 1a. In the ex vivo model, we observed a significantly higher mRNA expression at the highest dose of rhG-CSF stimulation (p = 0.01). We did not find any significant mRNA variation with the adjunction of anti G-CSF (figure 2a).

Thymidine Phosphorylase (TYMP), also known as the platelet-derived endothelial cell growth factor (PD-ECGF) or gliostatin, is a key angiogenesis promoting enzyme [20] as well as a cell migration promoter, especially by modulating integrin expression [21]. Its presence is also described in the endometrium [22]. In our infertile group, the TYMP endometrial mRNA expression was significantly lower (p = 0.0019), in those patients with either IF or RM (figure 1b). When the cultures were stimulated with the highest dose of rhG-CSF ex vivo, the TYMP endometrial mRNA expression appeared significantly higher (p = 0.04), as illustrated in figure 2b. G-CSF blocking antibody had no significant action on TYMP expression.

Integrin alpha-V/beta-3 (ITGB3), which is present in the endometrium [23], is described as being highly implicated in the cell migration and embryo implantation process [24]. It is a major endometrial adhesion molecule described as such not only in Human, but also in various animal models such as bovine [25] [26], porcine [27] [28] and ovine [29]. An ITGB3's down regulation has also been incriminated in pathological situations involving an impaired endometrial receptivity [30]. As an expected G-CSF endometrial target, ITGB3 mRNA expression was significantly enhanced (p = 0.001) by application, of the highest dose of rhG-CSF ex vivo (figure 2c), and without any change despite anti G-CSF adjunction. However, in this study, we could not obtain a significant difference in ITGB3 endometrial mRNA

expression between control and infertile patients, either with IF or RM. We thus tried to consider the possible interaction of ITGB3 with PLAUR.

Plasminogen Activator Urokinase Receptor (PLAUR), or uPAR (Urokinase Plasminogen Activator Receptor), is an essential actor in tissue remodelling through cell migration, proliferation and survival [31]. PLAUR is localised in the endometrium, its expression varying during the menstrual cycle [32]. It is described in early placental bed pregnancies and especially in those immune endometrial cells called uterine Natural Killers [33]. PLAUR is often implicated in impaired trophoblastic invasion, leading to pathological pregnancies with intra uterine growth retardation or pre eclampsia [34] [35] [36] [37]. Moreover, the PLAUR signalling pathway is described as requiring integrins, including ITGB3 [31]. Therefore, since we could not find any significant difference in endometrial PLAUR mRNA expression between control and infertile patients, we studied endometrial PLAUR and ITGB3 interaction by considering the following ratio: ITGB3 mRNA level / PLAUR mRNA level. When considering the ITGB3/PLAUR mRNA expressions (figure 1c), we observed a significant endometrial disequilibrium in these infertile patients with a history of RM (p = 0.02). When stimulated with the highest dose of rhG-CSF, PLAUR endometrial mRNA expression was significantly enhanced (p = 0.04), as illustrated in figure 2d. Again, these variations were not observed when adding anti G-CSF.

CD40 and its ligand CD40L are membrane molecules involved in the control of cellular proliferation via regulation of apoptotic mechanisms [38]. They are localised on immune cells, haematopoietic progenitors, epithelial cells and carcinomas [39]. When considering our previous microarray study, these genes seemed highly deregulated in the endometrium of infertile patients, and hypothetically interacting with G-CSF. In this study, CD40 endometrial mRNA expression did not significantly vary between control and infertile patients, when taken globally. But when considering the IF subgroup within the infertile patients, the CD40 mRNA expression was significantly lower compared to control group (p = 0.04), as shown in figure 1d. In the ex-vivo model, at the highest dose of rhG-CSF stimulation, CD40 mRNA expression only tend to be higher, without reaching significance (data not shown). CD40L mRNA expression level was very low in either control or infertile patients and did not vary in the ex-vivo culture with rhG-CSF supplementation. For both genes, we did not observe any significant mRNA variation with anti G-CSF adjunction.

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The mRNA expression variations for G-CSFR, TYMP, ITGB3 and PLAUR, observed under rhG-CSF stimulation, were found to be correlated, as shown in table 1, suggesting that the described pathways may be intricated.

To specifically study the action of endometrial rhG-CSF on its selected target genes, we used the same ex-vivo experiments with rhGM-CSF supplementation during 3 consecutive days. This control supplementation did not induce any variation on mRNA expressions of G-CSFR, TYMP, ITGB3 or PLAUR, as observed with rhG-CSF adjunction.

In all cases, adjunction of either anti G-CSF blocking antibody or anti GM-CSF blocking antibody did not show any variation in the target genes expression with the present ex vivo model. This may be due to existing redundant pathways or linked to the type of selected blocking antibody.

Conclusions

After showing the difference of expression of some hypothetical endometrial targets in infertile patients, and after observing the variations of expression of these target genes under specific rhG-CSF stimulation ex vivo, this study illustrates the putative key role of G-CSF during the embryo implantation process. This cytokine seems able to modulate fundamental genes intervening in the local embryo adhesion, cell migration, tissue remodelling and angiogenesis, unavoidable for a successful implantation and further placentation.

RhG-CSF appears actually as a promising innovative therapy in some difficult and unsolved cases of reproductive failure. However, strict indications of this supplementation in reproductive field are not established yet. Such expected endometrial actions may be tested after in vivo rhG-CSF supplementation and safety of such treatment still needs to be demonstrated on early stages of embryogenesis. Specific indications of pre conceptual rhG-CSF supplementation may also derive from diagnosed lack of endometrial expression of some of its target genes.

Acknowledgments

The authors thank all the patients without whom this study would not have been possible. We also thank all the involved physicians from ART Department in Pierre Rouques - Les Bluets Hospital. Figure 1: Endometrial mRNA expressions variation in control, IF and RM patients

IF: Implantation Failures

RM: Repeated Miscarriages

Results expressed in concentration ratio (Arbitrary Units) between target gene mRNA level and reference gene mRNA level

(*) Statistically Significant Difference, p<0.05

Figure 1a: G-CSFR mRNA expression variation in control, IF and RM patients

Figure 1b: TYMP mRNA expression variation in control, IF and RM patients

Figure 1c: ITGB3 and PLAUR mRNA expressions variation in control, IF and RM patients

Figure 1d: CD40 mRNA expression variation in control, IF and RM patients

Figure 1a: G-CSFR mRNA expression variation in control, IF and RM patients



Figure 1b: TYMP mRNA expression variation in control, IF and RM patients



Figure 1c: ITGB3 and PLAUR mRNA expressions variation in control, IF and RM patients



Figure 1d: CD40 mRNA expression variation in control, IF and RM patients



Figure 2: Endometrial mRNA expressions variation after rhG-CSF stimulation

Anti G-CSF: 3 days culture with G-CSF blocking antibody daily supplementation at 3 μg/ml G-CSF (1): 3 days culture with rhG-CSF daily supplementation at 20 ng/ml G-CSF (2): 3 days culture with rhG-CSF daily supplementation at 100 ng/ml G-CSF (3): 3 days culture with rhG-CSF daily supplementation at 200 ng/ml Results expressed in concentration ratio (Arbitrary Units) between target gene mRNA level after culture with and without specific stimulation, each patient being her own control

(*) Statistically Significant Difference, p<0.05

Figure 2a: G-CSFR mRNA expression variation after rhG-CSF stimulation Figure 2b: TYMP mRNA expression variation after G-CSF stimulation Figure 2c: ITGB3 mRNA expression variation after G-CSF stimulation Figure 2d: PLAUR mRNA expression variation after G-CSF stimulation

Figure 2a: G-CSFR mRNA expression variation after rhG-CSF stimulation



Figure 2b: TYMP mRNA expression variation after G-CSF stimulation



Figure 2c: ITGB3 mRNA expression variation after G-CSF stimulation



Figure 2d: PLAUR mRNA expression variation after G-CSF stimulation



Table 1: Correlation between target genes mRNA expression under G-CSF stimulation

Correlation between Target Genes Expression	Rho	p
ITGB3 & TYMP	31%	0,05
PLAUR & ITGB3	47%	0,003
PLAUR & TYMP	45%	0,005
GCSFR & ITGB3	67%	<0,0001
GCSFR & TYMP	46%	0,006
GCSFR & PLAUR	37%	0,02

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4. G-CSF Effects on a Pro Abortive Murine Model

4.1. The Pro Abortive Murine Model CBA/J x DBA/2

4.2. The Scheme of G-CSF Supplementation

4.3. Evaluation of Embryo Implantation and Embryo Resorption

4.4. Dedicated Original Article

4. G-CSF Effects on a Pro Abortive Murine Model



4.1. The Pro Abortive Murine Model CBA/J x DBA/2

Murine models exist to study embryo implantation and early pregnancy loss. The classical mating of CBA/J females with DBA/2 males displays an environment dependent high incidence of embryo resorption, which is not seen in the CBA/J females with Balb/c males control mating combination. The Embryo resorption in mice

is often, and classically, considered as equivalent to early pregnancy loss in Human. This mating combination has also recently been described as offering a murine model of preeclampsia.

Early pregnancy loss mechanism in this model is supposed to be non MHC linked and caused by seminal plasma paternal antigens interfering with the local endometrial immune environment, modulating the pre implantation endometrial biosensor. A recent publication on this precise subject is proposed in annexes.

In Assisted Reproductive Medicine G-CSF supplementation has been proposed as an effective treatment to reduce early pregnancy loss or increase embryo implantation rate, while its effects on embryo implantation itself remain poorly documented. Our aim was to identify the effects of G-CSF administration on subsequent embryo implantation and early pregnancy loss in these two crosses. We therefore tested the effects of systemic G-CSF supplementation at different doses in the two crossings.

4.2. The Scheme of G-CSF Supplementation

During post-ovulation and pre-implantation period, between day 1 and day 4 post coitum, intra-peritoneum injections of G-CSF were made to CBA/J female mice, after either the proabortive crossing (CBA/J x DBA/2) or the fertile crossing (CBA/J x Balb/c). The females, from either crossing were checked every morning for a vaginal plug and that day was designated as day 1 of gestation. Each primigravida female was then included in a specific supplemented group as described below (Figure 11).



Figure 11: Summary of the G-CSF murine supplementation scheme

Two G-CSF supplementation protocols were used: in the first one, three repeated G-CSF doses (diluted in 1% Bovine Serum Albumin) injected on days 1, 2 and 3 post-coitum, whereas the second one consisted of a single injection on the first day post-coitum (dpc).

Additionally, a group of CBA/J females from both crossing had the same scheme of injection as previously described, but performed with 1% Bovine Serum Albumin only, and formed the placebo injected groups. We finally formed two non-injected control groups for each crossing.



4.3. Evaluation of Embryo Implantation and Embryo Resorption

From Mouse Genome Informatics

Plugged females were sacrificed on day 10 of gestation. The uterine horns were opened, and the number of embryo implantation sites as well as the number of resorbed and resorbing embryos were evaluated in each group. The embryo resorption rate for each female was determined as the ratio between the number of resorpted implantation sites, and the number of total implantation sites, including ongoing and resorpted embryo implantation sites. The embryo implantation and embryo resorption rates were compared between females from

each crossing, for different G-CSF supplementation schemes, placebo or non-injected controls.

4.4. Dedicated Original Article

The experimental details and our results are described in the next dedicated article entitled "Variations of Embryo Implantation and Embryo Resorption Rates after Systemic Supplementation by Granulocyte-Colony Stimulating Factor in an Abortion Prone and Control Murine Mating Combinations", which was submitted to Journal of Reproductive Immunology.



"Research Article"

From Sempe, Le Petit Nicolas, 1960

Variations of Embryo Implantation and Embryo Resorption Rates after Systemic Supplementation by Granulocyte-Colony Stimulating Factor In an Abortion Prone and Control Murine Mating Combinations

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Abstract

In Assisted Reproductive Medicine, administration of Granulocyte-Colony Stimulating Factor (G-CSF) has been proposed as an effective treatment to reduce early pregnancy loss or increase embryo implantation rate, but its effects on embryo implantation itself remain poorly documented. Our aim was to identify the effects of G-CSF on subsequent embryo implantation and early pregnancy loss in a murine abortion prone mating combination as well as in control ones.

We therefore tested the effects of systemic recombinant G-CSF injection in the classical murine abortion model obtained by mating CBA/J females with DBA/2 males (CBA/J x DBA/2), displaying an environmental dependent high foetal resorption rate, and compared it to the classical relevant non resorption prone CBA/J x Balb/c combination. Recombinant G-CSF was injected to CBA/J female mice, in the 2 mating combinations, at different gestation days and different doses during pre-implantation period.

At day 10 post-coitum, the embryo implantation rates and early embryo resorption rates (classically considered as good equivalents of an early pregnancy loss in Human) were compared to those observed in the control groups receiving placebo injections or in the non-injected control groups.

High doses of G-CSF significantly increased embryo implantation sites in both models, but also increased the embryo resorption rate in the pro-abortive mating. Low doses of G-CSF decreased the number of implantation sites, but nevertheless also decreased the resorption rate in this abortion prone mating combination. In the control, non abortive mating, those same low doses of G-CSF decreased implantation sites without modifying the resorption rate.

Since the implantation rates are modified by injection of recombinant G-CSF, we concluded that it might affect embryo selection by modifying the endometrial biosensor in these murine models. However, the timing and the dose of injection are crucial. This observation might not be trivial but rather highlights the necessity to define specific indications of G-CSF administration through a better understanding of the local mechanisms induced.

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Keywords

Granulocyte-Colony Stimulating Factor, Embryo Implantation, Murine Model, Early Pregnancy Loss, Endometrial Biosensor

1. Introduction

Granulocyte-Colony Stimulating Factor (G-CSF), or CSF-3, is one of the key cytokines acting on proliferation and differentiation of leukocytes, studied from the mid 1960's. Its recombinant form is used since late 1980's in haematology for treatment or prevention of chemotherapy induced neutropenia [1], congenital agranulocytosis [2] or haematopoietic stem cell transplantation [3].

The involvement of Colony Stimulating Factors (CSFs) in reproduction was raised from early 1970's with the demonstration of stimulatory effect of placental media on hematopoietic cell multiplication, in mouse [4] and Human [5]. After these primary descriptions and their localisation in the reproductive tract [6] [7], various research teams highlighted the immuno-trophic [8] [9], anti-apoptotic [10] [11] and immuno-modulatory [12] [13] actions of the CSFs during the early steps of pregnancy.

Today, recombinant human G-CSF (rhG-CSF) is one of the major emergent innovative therapies in reproductive medicine, suggested in disorders such as dysovulation [14] [15], recurrent miscarriages [16] or embryo implantation failures [17] [18]. It is used is either by systemic subcutaneous injections or by local intra-uterine infusions. The majority of these clinical trials are evaluating rhG-CSF supplementation's effects on embryo implantation and early pregnancy loss [19] [20] [21] [22], although its mechanisms of action on the early stages of implantation have never been clearly established.

In this respect, murine models exist to study embryo implantation and early pregnancy loss. The classical mating of CBA/J females with DBA/2 males [23] displays an environment dependent high incidence of embryo resorption (25 to 40%), which is not seen in the CBA/J x Balb/c control mating combination, (displaying an embryo resorption rate of 5 to 15%). The Embryo resorption in mice is often, and classically, considered as equivalent to early pregnancy loss in Human. This mating combination has also recently been described as offering a murine model of pre-eclampsia [24].

Our aim was to identify the effects of administration, of G-CSF on subsequent embryo implantation and early pregnancy loss in these two crosses. We therefore tested the effects

of systemic recombinant murine G-CSF (rmG-CSF) supplementation at different doses in the two matings. In each cross, the results were compared to a group receiving placebo injections. This supplementation was performed at the post ovulation and pre-implantation periods, to specifically study the effects of rmG-CSF precisely on the endometrial biosensor, distinguishing it from the possible effects on ovulation or from the known specific actions on embryonic growth itself.

2. Material and methods

2.1. Murine Crossings

All experiments were carried out in the animal quarters of INSERM U782 (32 rue des Carnets, 92140, Clamart, France). All mice, i.e. 102 CBA/J females (H-2k), 5 DBA/2 and 5 Balb/c males (both H-2d), were obtained from Charles River, France, at the age of 6 weeks for females and 8 weeks for males. All animals were maintained for one week in the animal quarters before mating. Matings were performed as trios: one male and two females per cage. The females, either crossed with DBA/2 or Balb/c males, were checked every morning for a vaginal plug and that day was designated as day 1 of gestation. Each primigravida female was then included in a specific supplemented group as described below.

2.2. G-CSF Supplementation and control groups

During post-ovulation and pre-implantation period, between day 1 and day 4 post coitum, intra-peritoneum (IP) injections of recombinant murine G-CSF (rmG-CSF) were made to CBA/J female mice, after either the pro-abortive crossing (CBA/J x DBA/2) or the fertile crossing (CBA/J x Balb/c).

Two rmG-CSF injection protocols were used: in the first one , three repeated doses of 50 ng of rmG-CSF (R&D, France) injected on days 1, 2 and 3 post-coitum, whereas the 2nd one consisted of a single injection of 50 ng of rmG-CSF on the first day post-coitum.

For injection, 50ng of rmG-CSF were diluted in a 1% Bovine Serum Albumin solution (Sigma-Aldrich, France). The total volume injected was 0.3 ml. The four supplemented groups were formed as follows: 7 CBA/J females from the fertile crossing and 8 CBA/J females from the pro-abortive crossing had the three repeated IP rmG-CSF protocol. 13 CBA/J females from each crossing had the single IP rmG-CSF protocol.

Additionnlly, a group of CBA/J females from both crossing had the same scheme of injection as previously described, but perfo0rmed with 1% Bovine Serum Albumin (BSA) only , and

formed the four placebo injected groups. 13 CBA/J females from each crossing had a single IP injection of 0.3 ml of 1% BSA on day 1 post-coitum. 7 CBA/J females from each crossing had a series of 3 IP injections of 0.3 ml of 1% BSA on days 1, 2 and 3 post-coitum. All injection, including rmG-CSF and placebo, were performed by the same operator.

We finally formed two non-injected control groups: 11 CBA/J females from the control crossing and 10 CBA/J females from the pro-abortive crossing.

2.3. Embryo implantation and resorption rate evaluation

Plugged females were sacrificed on day 10 of gestation by cervical dislocation. The uterine horns were opened, and the number of embryo implantation sites, as well as the one of resorbed and resorbing embryos resorption sites, were evaluated in each group. The embryo resorption rate for each female was determined as the ratio between the number of resorbed implantation sites, and the number of total implantation sites, including ongoing and resorbed embryo implantation sites [23] [25].

2.4. Statistical Analysis

Statistical analysis was performed with Statview[®] and Medcalc[®] software, using the ANOVA test. The results are given as mean (+/- SD) for each group. A p value <0.05 was considered significant.
3. Results

3.1. Control embryo implantation and resorption rates

The basic embryo resorption rates, in the conditions of our study, and in these animal quarters, were 10.4 % for the non-abortive crossing CBA/J x Balb/c and 24.9 % for the pro-abortive crossing CBA/J x DBA/2. The number of total implantation sites was respectively 5.5 and 8.5.

3.2. Embryo implantation and resorption rates in mice receiving a series of three injections of rmG-CSF or placebo

In the non abortive crossing, both repeated IP injections of rmG-CSF and placebo, increased the total number of embryo implantation sites (means of respectively 10.8 and 9.4 sites versus 5.5 in the non-injected group) This raise was statistically significant in both injected groups when compared to the non-injected group (p=0.01 for the placebo group, p=0,0002 for the rm-G-CSF group). However, both repeated injections also increased significantly at the same time the embryo resorption rate (respectively 35.4 % and 31.0 % versus 10.4 % in the non-injected group, p=0.02). However, there were no significant differences between rmG-CSF or placebo repeated injections, be it for the number of embryo implantation sites or the embryo resorption rates.

In the abortion prone crossing, the repeated injections of rmG-CSF increased embryo implantation sites when compared with the placebo or the non-injected control group (12.8 versus 9.4 sites, p=0.01). When considering embryo resorption rate, it was significantly increased with repeated injections of rmG-CSF while only tending to be higher with repeated placebo injections (48.1 % with rmG-CSF versus 34.1 % with placebo, p=0.007).

3.3. Embryo implantation and resorption rates in mice receiving a single injection of rmG-CSF or placebo

In the pro-abortive crossing, a single injection of rmG-CSF at day 1 post-coitum very significantly decreased the total number of implantation sites (5.3 sites with rmG-CSF versus 11.0 sites with placebo, p<0.0001) (Figure 1) and also significantly decreased resorption rate (9.6 % with rmG-CSF versus 31.0 % with placebo, p<0.0001) (Figure 2).

Unexpectedly, in the fertile crossing, the very same unique injection of rmG-CSF, significantly decreased implantation sites (2.6 sites with rmG-CSF versus 6.5 sites with placebo, p=0.0007) without modifying the resorption rate, but it should also be reported that more than 60 % of CBA/J plugged females, did not display any sign of embryo implantation (8/13).

An overview of the results is shown in Table 1.

4. Discussion

The chosen murine mating combinations have been since 1983 used in a number of independent laboratories as a model of early pregnancy loss, equivalent to idiopathic recurrent miscarriage in Human. Early pregnancy loss mechanism in this model is supposed to be non MHC linked, since both paternal strains are H-2d. This mechanism is believed to be caused by different paternal antigens provoking a protective (by Balb/c) or an abortion inducing (by DBA/2) immune response [26]. Seminal plasma antigens are involved and it has been suggested that these antigens could locally modulate the endometrial environment via regulatory cells T and dendritic cells [27] [28] [29], turning locally the endometrial environment into a protective one. The endometrial biosensor intervention is therefore crucial in this murine model.

The data obtained in this system, as well as the ones obtained by very early complement neutralisation [24] [30] suggest strongly that the shift between an abortion doomed pathway or another leading to successful pregnancy occurs very early, much earlier than originally thought in the 90s.

These observations led us to choose an early timing for rmG-CSF supplementation: rm-G-CSF supplementation from day 1 to day 3 of gestation, implies that we had no action on earlier events (ovulation) or later ones (embryonic growth), the murine embryo implantation occurring on day 4 of gestation. This is important since ovulation [14], placental and embryonic growth [31] [32] are also described to be highly influenced by Colony Stimulating Factors. Our purpose was thus to target the early pre-implantation endometrial biosensor.

In this study, our basic embryo resorption rate was 10% for the CBA/J x BALB/c control mating, which, though such basal rates have been reported in various laboratories, happened to be higher than the usual 5% rate classically described in our breeding conditions since the original publication [23], albeit in some series we have occasionally observed such rates. This is not totally surprising since the resorption rates are known to be environment dependent, and variations in diet, for example, can cause such variations.

Anyhow, all the statistical comparisons were made considering this actual resorption rate, obtained in this study's specific conditions.

When considering repeated injections of either rmG-CSF or placebo, even in the fertile crossing, the embryo resorption rates seem very high. This observation could be linked to the well-known stress induced murine pregnancy loss [33] [34], the stress, in this case, occurring from repeated injections themselves.

However, when focusing on the pro-abortive subgroup receiving higher doses of rm-CSF, we noticed a larger number of embryo implantation sites, even though there was a higher embryo resorption rate when compared to the placebo, testifying for an alteration of the endometrial biosensor by rmG-CSF.

When examining the case of CBA/J females from the pro-abortive crossing which received a single rmG-CSF injection, we found a lightly beneficial effect of this supplementation when compared to the placebo. While reducing the number of embryo implantation sites, this supplementation scheme also reduced the embryo resorption rate. But this supplementation did not result in a significantly higher ongoing embryo sites in mice receiving G-CSF.

Finally, if we consider the group of CBA/J females from the control crossing which received a single rmG-CSF injection to the ones which received a single injection of placebo, we observed the same embryo resorption rate but with a surprisingly reduced number of implantation sites, and a large portion of mice without any embryo implantation.

To summarize, we found that, in this specific murine model, the endometrial biosensor can be modulated by rmG-CSF systemic supplementation, with variable effects when considering the rm-G-CSF doses and the type of crossing. Particularly, when considering the fertile murine model, this supplementation does not appear necessarily harmless.

5. Conclusions

In these murine models, G-CSF seems to affect the uterine embryo selection by modifying the endometrial biosensor, the injection being performed after the ovulation but before the embryo implantation. G-CSF supplementation effects on embryo implantation and resorption depend on the doses and the injection timing. These effects are different when considering control or abortive murine models. G-CSF supplementation has been described as promising to reduce early pregnancy loss or increase embryo implantation rate in case of pathology. Timing and dose of injection seem therefore crucial. Moreover, this supplementation does not seem trivial when considering fertile cases and highlights the necessity to define specific indication through a better understanding of the induced local mechanism.

Acknowledgements

We thank Mr Fabrice Petit for providing helpful technical advice.

Table 1: Summary of Main Results

Results given as mean (+/- SD) for each group

Embryo resorption rate: Number of resorption sites / total of implantation sites (resorpted + ongoing implantation sites)

3 IP G-CSF: Repeated intra-peritoneal injection of rmG-CSF on days 1, 2 and 3 of gestation

3 *IP BSA:* Repeated intra-peritoneal injection of Bovine Serum Albumin solution on days 1,2 and 3 of gestation (placebo group)

1 IP G-CSF: Single intra-peritoneal injection of rmG-CSF on day 1 of gestation

1 *IP BSA:* Single intra-peritoneal injection of Bovine Serum Albumin solution on day 1 of gestation (placebo group)

0 IP: Non-injected control group

PRO-ABORTIVE CROSSING CBA/J female x DBA/2 male FERTILE CROSSING CBA/J female x BALB/c male

Protocol group	3 IP G-CSF	3 IP BSA	1 IP G-CSF	1 IP BSA	0 IP	3 IP G-CSF	3 IP BSA	1 IP G-CSF	1 IP BSA	0 I P
Number of CBA females	7	7	13	13	11	8	7	13	13	10
Total implantation sites	10.8 (3.2)	9.4 (3.2)	2,6 (3.5)	6,5 (1.9)	5.5 (1.6)	12.8 (2.8)	9.4 (2.0)	5,3 (3.8)	11,0 (2.5)	8.5 (2.2)
Ongoing implantation sites	6.7 (1.6)	6.0 (1.2)	2,3 (3.0)	5,5 (1.4)	5 (1.6)	6.6 (1.5)	6.1 (1.3)	4,7 (3.4)	7,5 (1.6)	6.3 (1.4)
Resorpted implantation sites	4.1 (2.4)	3.4 (2.3)	0,3 (0.7)	1.0 (1.4)	0.5 (1.4)	6.2 (1.8)	3.2 (1.1)	0,5 {0.8}	3,5 (1.7)	2.2 (1.1)
Embryo resorption rate	35.4 %	31.0 %	10.0%	12,6%	10,4 %	48.1%	34.1%	9'6 %	31.0%	24.9 %
CBA without embryo implantation	0	2	8	0	0	0	0	4	0	0

Figure 1: Total implantation sites in plugged CBA females which received a single injection of rmG-CSF or placebo, after abortion prone or non abortive crossing

BALB BSA: Single intra-peritoneal injection of BSA solution (placebo) on day 1 of gestation after Balb crossing BALB GCSF: Single intra-peritoneal injection of rmG-CSF on day 1 of gestation after Balb crossing DBA BSA: Single intra-peritoneal injection of BSA solution (placebo) on day 1 of gestation after DBA crossing DBA GCSF: Single intra-peritoneal injection of rmG-CSF on day 1 of gestation after DBA crossing



Figure 1: Embryo resorption rate in plugged CBA females which received a single injection of rmG-CSF or placebo, after abortion prone or non abortive crossing

BALB BSA: Single intra-peritoneal injection of BSA solution (placebo) on day 1 of gestation after Balb crossing BALB GCSF: Single intra-peritoneal injection of rmG-CSF on day 1 of gestation after Balb crossing DBA BSA: Single intra-peritoneal injection of BSA solution (placebo) on day 1 of gestation after DBA crossing DBA GCSF: Single intra-peritoneal injection of rmG-CSF on day 1 of gestation after DBA crossing



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5. Discussion, Conclusion and Perspectives

5.1. G-CSF effects on Human Endometrium

5.1.1. G-CSF endometrial target genes

5.1.2. Target genes expression in fertile women and infertile patients' endometrium 5.1.3. Endometrial target genes variation under ex vivo G-CSF supplementation

5.2. G-CSF effects on a Pro Abortive Murine Model

5.3. Conclusion and Perspectives

5. Discussion, Conclusion and Perspectives

5.1. G-CSF effects on Human Endometrium

5.1.1. G-CSF endometrial target genes

Granulocyte-Colony Stimulating Factor endometrial targets we selected from our previous microarray study [144] have various functions involved in embryo implantation process.

G-CSF receptor (G-CSFR) has been localised at the maternal foetal interface [145], with cyclic and gestational regulations [146] [147] and a trophoblast growth promoting role [148].

Thymidine Phosphorylase (TYMP), localised in the endometrium [149], is a key angiogenic enzyme [150] as well as a cell migration promoter, especially by modulating integrin expression [151].

Integrin alpha-V/beta-3 (ITGB3), also previously localised in the endometrium [152], is described as being highly implicated in the cell migration and embryo implantation process [153]. It is a major endometrial adhesion molecule described whose down regulation has also been incriminated in pathological situations involving an impaired endometrial receptivity [154].

Plasminogen Activator Urokinase Receptor (PLAUR) is an essential actor in tissue remodelling through cell migration, proliferation and survival [155]. Its signalling pathway is described as requiring integrins interaction, including ITGB3 [155]. PLAUR expression is localised in the endometrium, varying during the menstrual cycle [156], in early placental bed pregnancies and especially in uterine Natural Killer cells [157]. PLAUR is implicated in impaired trophoblastic invasion, leading to pathological pregnancies with intra uterine growth retardation or preeclampsia [158] [159] [160] [161].

CD40 and its ligand CD40L are membrane molecules involved in the control of cellular proliferation via regulation of apoptotic mechanisms [162]. They are localised on immune cells, haematopoietic progenitors, epithelial cells and carcinomas [163]. When considering

our previous microarray study [144], these genes seemed highly deregulated in the endometrium of infertile patients, and hypothetically interacting with G-CSF.

5.1.2. Target genes expression in fertile women and infertile patients' endometrium

When considering fertile women or infertile patients with idiopathic repeated miscarriages (RM) or idiopathic repeated embryo implantation failure (IF), we noticed significant variations in the endometrial expression of the different target genes previously selected.

As a reminder, TYMP endometrial mRNA expression was significantly lower in infertile patients, with either IF or RM, when compared with fertile patients.

G-CSFR and CD40 endometrial mRNA expression were significantly lower in the subgroup of patients with IF when compared with fertile women or patients with RM.

When observing ITGB3 or PLAUR individually, we did not notice any endometrial mRNA expression between control and infertile patients, either with IF or RM. We thus tried to consider the possible interaction of ITGB3 with PLAUR. We studied endometrial PLAUR and ITGB3 interaction by evaluating the following ratio: ITGB3 mRNA level / PLAUR mRNA level. When considering the ITGB3/PLAUR mRNA expressions, we observed a significant endometrial disequilibrium in the infertile patients with a history of RM, when compared to fertile women or patients with IF.

5.1.3. Endometrial target genes variation under ex vivo G-CSF supplementation

In the ex vivo model, the significant mRNA expression variations were observed at the highest dose of G-CSF stimulation. The mRNA expression was significantly higher with G-CSF stimulation for G-CSFR, TYMP, ITGB3 and PLAUR. CD40 mRNA expression tend to be higher under this ex vivo stimulation, without reaching significance, and CD40L mRNA level did not vary.

These reported variations were specific to G-CSF and did not happen when the same experiments were performed with Granulocyte Macrophage Colony Stimulating Factors (GM-CSF).

For all target genes, adjunction of anti G-CSF blocking antibody did not provoke any variation in their expression with the present ex vivo model. This may be due to existing redundant pathways or linked to the type of selected blocking antibody.

The mRNA expression variations for G-CSFR, TYMP, ITGB3 and PLAUR, observed under G-CSF stimulation, were found to be correlated, suggesting that the described pathways may be intricated.

In Human endometrium during the window of implantation, after showing the difference of expression of some hypothetical targets in infertile patients, and after observing the variations of expression of these target genes under specific G-CSF stimulation ex vivo, our study illustrates the key role of G-CSF during the embryo implantation process. This cytokine seems able to modulate fundamental genes intervening in the local embryo adhesion, cell migration, tissue remodelling and angiogenesis, unavoidable for a successful implantation and further placentation.

However, the observed variations under G-CSF stimulation on the expression of selected genes were only shown on ex vivo endometrial implants, thus in artificial conditions. Secondary pathways and complementary redundant systems should also be considered. In vivo endometrial response to G-CSF supplementation may vary from what is expected given the results observed in culture.

5.2. Granulocyte-Colony Stimulating Factor (G-CSF) effects on a Pro Abortive Murine Model

The murine mating combination CBA/J female x DBA/2 male is a model of early pregnancy loss, compared with the fertile crossing CBA/J female x Balb/c male. Embryo resorption sites observed on murine uterine horns reflect early pregnancy loss and a high embryo resorption rateis equivalent to idiopathic recurrent miscarriage in Human.

Early pregnancy loss mechanism in this model is supposed to be non MHC linked. This mechanism is believed to be caused by different paternal antigens provoking a protective (by Balb/c) or an abortion inducing (by DBA/2) immune response [164]. Seminal plasma antigens are involved and it has been suggested that these antigens could locally modulate the endometrial environment via regulatory cells T and dendritic cells [165] [166]. The endometrial biosensor intervention is therefore crucial in this murine model.

The G-CSF supplementation scheme we described was chosen to specifically target the preimplantation endometrium, avoiding supplementary on earlier ovulation or further placental or embryonic growth.

When analysing our data on embryo implantation and embryo resorption variations, we took into consideration the fact that these rates were dependent on animals' environment, such as diet, cages localisation or the stress induced by repeated injections [167] [168].

Focusing on the pro-abortive subgroup receiving higher doses of G-CSF, we noticed a larger number of embryo implantation sites, even though there was a higher embryo resorption rate when compared to the placebo.

Examining the case of CBA/J females from the pro-abortive crossing which received a single G-CSF injection, we found a beneficial effect when compared to the placebo. While reducing the number of embryo implantation sites, this supplementation scheme reduced the embryo resorption rate.

Finally, if we consider the group of CBA/J females from the control crossing which received a single rmG-CSF injection, we observed the same embryo resorption rate but with a

surprisingly reduced number of implantation sites, and a large portion of mice without any embryo implantation.

In the specific murine model we studied, the various effects we observed on embryo implantation and embryo resorption rates, in abortion prone or fertile murine crossing, testify for a modulation of the endometrial biosensor by G-CSF supplementation. The endometrial effect of G-CSF administration seems to depend on the dose and timing. Particularly, when considering the G-CSF induced effects on our fertile murine model, this supplementation does not appear necessarily harmless.

Nevertheless, we have to remind that the results obtained with the chosen murine crossings, even if they are considered as an approaching model to recurrent miscarriages, are not directly transposable to Human. This is not only linked to the obvious differences in embryo implantation mechanisms between the two species, but also to the high environmental susceptibility of mice in the reproductive field.

5.3. Conclusion and Perspectives

Colony Stimulating Factors (CSFs) family is an illustration of the scope of reproductive immunology. These immune cytokines and their receptors are localised along the reproductive tract, and not solely in immune cells. Through various effects on local inflammation, immune modulation or immunotrophicity, they act on reproductive functions such as ovulation, embryo implantation, placentation and embryo development (Figure 12). Fundamental studies on CSFs, their localisation, their physiological or pathological variations, their various actions, have generated direct applications in reproductive medicine.





GM-CSF: Granulocyte-Macrophage Colony Stimulating Factor **G-CSF**: Granulocyte-Colony Stimulating Factor **M-CSF**: Macrophage-Colony Stimulating Factor As already described, many CSF supplementation protocols are under evaluation in patients with history of reproductive failure. The medical indications are numerous, including idiopathic repeated miscarriages, unexplained embryo implantation failure or unresponsive endometrial trophic defects. And the supplementation schemes are even more variable: local or systemic supplementation, variable doses, variable timing of administration.

Despite their undeniable interest in reproductive medicine, CSFs actions are still not elucidated, especially concerning long term effects of CSFs used on early stages of embryo development.

Besides, a wrong endometrial timing induced in the pre-implantation process might interfere with a local convenient immune environment and turn prejudicial for embryo implantation and development.

Our murine results, even if not directly transposable to cases of Human recurrent miscarriages, illustrate a considerable endometrial effect of the systemic G-CSF supplementation.

To refine our knowledge on the molecular endometrial effects of G-CSF in this particular model, we could consider further transcriptomic studies on pre-implantation decidual samples, in mice with or without G-CSF supplementation, either systemic or by local infusion.

Granulocyte Colony Stimulating Factor (G-CSF) in particular, appears actually as a promising innovative therapy in some difficult and unsolved cases of reproductive failure, given its action on fundamental mechanisms regulating embryo implantation process (Table 2). However, strict medical indications of this supplementation in reproductive field and its scheme of administration are not established yet.

 Table 2: Summary of the major G-CSF target genes in Human endometrium and their

 actions on embryo implantation process

Major Endometrial Events In Implantation Process	Involved Endometrial G-CSF Target Genes
Embryo Adhesion	TYMP ITGB3
Cell Migration	TYMP ITGB3 PLAUR
Cell Proliferation	PLAUR CD40
Angiogenesis	ТҮМР

G-CSF: Granulocyte-Colony Stimulating Factor, **TYMP**: Thymidine Phosphorylase, **ITGB3**: Integrin alpha-v/beta3, **PLAUR**: Plasminogen Activator Urokinase Receptor

In our present studies, we considered pre implantation decidualised endometrium in nonconceptual cycles, without the presence of the embryo, and we identified specific endometrial dysregulations. These observations imply that the pre conceptual and preventive diagnosis of some local dysregulations is possible.

Given the complexity of the locally involved mechanisms, specific and precise diagnoses seem necessary. Furthermore, developing pre conceptual endometrial biomarkers may help in this purpose.

Developing pre-conceptual biomarkers would not only help in the diagnostic field, but can be of a great interest to discern therapeutic indications.

Applying our observations to the emerging but fundamental concept of the endometrial biosensor, we can consider that targeted therapeutics may improve reproduction via the

pre conceptual recovery of a receptive endometrium. Specific indications of G-CSF supplementation in case of reproductive failure may derive from the primary pre conceptual diagnosis of the local dysregulation of some of its endometrial targets.

On the fundamental side, comparing the variations of these endometrial biomarkers in patients under G-CSF stimulation would also confirm our ex-vivo observations.

Optimising endometrial receptivity should be of a major interest and part of the future therapeutics, not only specifically in reproductive medicine, but in longer term, by preventing further obstetrical pathologies that may result from an initial defective embryo implantation.

6. References



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7. Annexes

7.1. Reference Article for the Endometrial Microhistoculture

7.2. Reference Article for the Endometrial Microarray Study

7.3. Reference Article for the Pro Abortive Murine Model

7.4. Summary in French

7.1. Reference Article for the Endometrial Microhistoculture

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TWEAK Appears as a Modulator of Endometrial IL-18 Related Cytotoxic Activity of Uterine Natural Killers

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Abstract

Background: TWEAK (Tumor necrosis factor like WEAK inducer of apoptosis) is highly expressed by different immune cells and triggers multiple cellular responses, including control of anglogenesis. Our objective was to investigate its role in the human endometrium during the implantation window, using an ex-vivo endometrial microhistoculture model. Indeed, previous results suggested that basic TWEAK expression influences the IL-18 related uNK recruitment and local cytotoxicity.

Methodology: Principal Findings: Endometrial biopsies were performed 7 to 9 days after the ovulation surge of women in monitored natural cycles. Biopsies were cut in micro-pieces and cultured on collagen sponge with appropriate medium. Morphology, functionality and cell death were analysed at different time of the culture. We used this ex vivo model to study mRNA expressions of NKp-16 ta uNK cytotoxic receptor) and TGF-beta1 (protein which regulates uNK cytokine production) after adjunction of excess of recombinant IL-18 and either recombinant TWEAK or its antibody. NKp46 protein expression was also detailed by immunohistochemistry in selected patients with high basic mRNA level of IL-18 and either low or high mRNA level of TWEAK. The NKp46 immunostaining was stronger in patients with an IL-18 over-expression and a low TWEAK expression, when compared with patients with both IL-18 and TWEAK high expressions. We did not observe any difference for TWEAK or its antibody was added in the culture medium. In a pro-inflammatory environment (obtained by an excess of IL-18), inhibition of TWEAK was able to increase significantly NRp46 and TGF-beta1 mRNA expressions.

Conclusions/Significance: TWEAK doesn't act on IL-18 expression but seems to control IL-18 related cytotoxicity on uNK cells when IL-18 is over-expressed. Thus, TWEAK appears as a crucial physiological modulator to prevent endometrial uNK cytotoxicity in human.

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Conclusions/Significance: TWEAK doesn't act on IL-18 expression but seems to control IL-18 related cytotoxicity on uNK cells when IL-18 is over-expressed. Thus, TWEAK appears as a crucial physiological modulator to prevent endometrial uNK cytotoxicity in human.

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Introduction

The endometrium is remodelled throughout the menutrual cycle and exhibits only a short period of receptivity, known as the 'implantation window', which is crucial both for implantation and gestation and still remains poorly explored in routine reproductive medicine. Endometrium becomes receptive to blattocyst implantation 6 to 0 days after coulation and remains receptive for 4 days (cycle days 20-20) [1]. Such differentiation of the matternal compartment, under hormonal mutuol, is essential to allow stromal cells to acquire the unique ability to regulate trophoblast invasion, to resist inflammatory and oxidative insults, and to dampen local maternal immune response. In humans, decidualiration of the stromal compartment occurs in the mid-luteal phase of the menstrual cycle, independently of pregnancy, in contrast to most of animal models. This raises the possibility that biochemical analysis of timed endometrial biopsy samples taken in a nonconception cycle could be informative of subsequent pregnancy outcome [2].

It has been proposed that uterine natural killer cells (uNK) could exert, directly or not, a positive or negative control of the early steps of implantation [3], [4]. These cells can success and control an array of cytokines which are important in angiogenesis, placental development and in pregnancy establishment. A dyaregulation of these cytokines could be in part responsible of embryo implantation failures. In previous studies, we reported that IL-15 and IL-18 expressions were significantly different in patients who failed to implant when compared with fertile control individuals [5]. We also observed that IL-15 and IL-18 expressions were correlated with the local uNK (CD56+) recruitment and unbendometrial angiogenesis as reflected by the vascular flow index quantified by 3-D altrasound with angiography [6].

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TWEAK (Tumor necrosis factor like WEAK inducer of apoptosis) is a transmembrane protein, which can be cleaved to function as a soluble cytokine [7]. It's highly expressed by immune cells type (monocytes, dendritic cells, natural killers cells...) and many tissues [8].

First described as a weak apoptosis inducer, TWEAK triggers multiple cellular responses [9] ranging from proliferation to cell death, including control of angiogenesis [10], [11,12]. TWEAK has also been described as a partner to TNF (Tumor Necrosis Factor) playing a « Yin and Yang » function in immunity [13].

We recently reported that TWEAK and IL-10 mRNA expression were correlated in patients with implantation failures [14]. Therefore we investigated whether TWEAK regulates IL-10 expression or at the opposite if IL-10 acts on TWEAK expression, TWEAK adRNA and protein expression does not show variations through the menutrual cycle.

However in basial level of expression influence the IL-18 related uNK tecruitment and local cytotoxicity. Indeed, IL-18 is a breakent cytokine which can promote local angiogenesis and immunotrophiam at appropriate dose but conversely promote a cytotoxic and thus deleterious commitment of uNK cells if present in excess [3]. Such documentation appears to be essential to define the state of uterine receptivity.

Endometrial dispersed cells cannot be used to document the in ris environment since this technique does not maintain the interactions between epithelial cells, arounal cella, endothelials cells and immune cells (uterine natural killer cells, T regulatory cells and dendritic cells). The development of a human et rise model in which the complexicity of endometrium is preserved as well as its functionality is an absolute requirement. In order to study strongepithelial interactions in the human endoenetrium, many authors elaborated culture conditions recombining separated stromal and epithelial cells in different extracellular matrices [15,16,17,18]. In ach models, epithelial cells have been cultivated as monolayers on top of matrigel and stromal cells beneath them on plastic or enhedded in collagen or Matrigel. Other methods were described using polycarbonate or nitrocellulose culture of tissue insert [19,20] and demonstrated human epithelial and stromal cells were viable during 1 to 5 days. In all these models, cells viability was demonstrated but neither of them appears fully appropriate to study the expression and production of cytokines or growth factors in endometrial cells as well as to mimick the complexicity of their interactions with immune and endothelials cells.

We thus decided to develop a model of endometrial microbinized/use, adapted from the one elaborated by Meau et al [21] for the placenta, in which the complex interactions between epithelial, stromal and immune crils were preserved and the viability and responsiveness of the cells are verified. Using this model, we investigated whether TWEAK influences the activities of uNK cells by a modification of IL-10 expression or function.

Results

Validation of microhistoculture model

Cells morphology: The main problem we encountered with endowersions is are histocolure was to maintain differentiated cells and avoid they become fibrobiantic. We analyzed the endometrium structure as well as the morphology of cells by immanohistochemistry using two different antibodies; vimentin (endotticical cells staming) and KL1 cytokentm (epithetial cells staining). We compared the morphology at different days of culture with or without estradiol and progesterone addition (50 mmol/l) and with or without use of collagen sponge (Figure 1). In culture without sponge use, stronal cells are flat and clongated (Figure 1C and D) compared to those cultured with sponge which remained rounded (Figure 1E and F). But in both case epittetial cells are dispersed. Cells cultured with estradiol and progesterone (Figure 1G J) displayed a well perserved morphology, where epithelial cells maintain their polarisation. We realised the same experience using endometrian biopsies from proliferative phase and observed the same results (data not shown). Thus, to conserve their morphology endometrial cells must be cultured on collagen sponge with hormones addition in mediam.

Cells death: Cells viability was evaluated by immunohimchemistry using Annexis V Fluos, an antibody coupled to FTIC (Figure 2). The Annexis V assay provides effective method to detect apoptosis at a very early stage. After the induction of spoptosis phosphatidyherine is translocated from the inner (symplasmic) leaflet of the membrane to the outer (sell surface) leaflet, phosphatidyherine on the outer leaflet is available to bind liabeled Annexis V, providing the basis for a simple staining asay.

After two days of culture we only observed Annexia V staining on cells growing without collagen aponge matrix (Figure 2A and B) compared to those culture with Gelliann aponge (Figure 2C and D). After five days, all cells cultured with or without sponge are labeled (Figure 2E–FI). Besides, after five days of culture without sponge cells appeared elongated and fibeoblastic whereas cells growing with collagen spinge matrix are round.

We didn't observe any difference on cells viability when we added estradioi and progesterone in culture mediam.

Cells functionality: We assay in supernatants, by Luminex Technology, after 2 and 3 days of culture 8 cytokines and growth factors: interleukine 1B (IL-Ib), interleukine 1 receptors: (IL-IRa). granulocyte colony stimulating factor (G-CSP), interferon inducible protein-10 kilodaltons (IP-10), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1ß (MIPIb), platefet-derived growth factor \$ (PDGFb) and regulated on activation of normal T cell expressed and secreted (RANTES). These molocules were chosen as a marken a of endometrial activity cells because their expression were different between flushing with endometrial cells and flushing with no cells (data not shown). We previously observed that addition of estradiol and progesterone are autable to maintain a well preserve structure of epithelial cells. Our objective was to validate a model to study expression or production of bio-molecules. So it was important to determine whether estradiol and progesterone supplementation influence cytokines or growth factors production.

We realised eight different conditions using 3 estradiol or progesterone concentrations (50 nmol/1, 100 nmol/1, 200 nmol/1) and a control condition without hormone. Cytokines and growth factors production was assayed after 2 and 3 days of culture. Because the cellular mass from endometrium sampling could not he standardised, cytokine/growth factor production by day could not be compared between individuals. So we chose to express our results by the ratio day 3/day 2 cytokines production. This ratio was measured by patients for each cytokine and each hormonal concentration compared to control (without hormones). No significant variation of cytokines and growth factors production was observed according to distinct concentrations of estradiol or progesterone or their comparison to control condition. Figure 3 represents the cytokine/growth factor production for minimal concentration of entradiol and progesterone (50 nmol/1) for each patient.

Role and regulation of TWEAK in endometrial cells

A minimal concentration (50 nmol/l) of enradiol and progeterone was added in each culture condition for all the experiments.

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Figure 1. Endometrium cells morphology providing from luteal phase. Revealed by immunohistochemistry with cytokeratin (60) and vimentin antibodies (x 6.6). A-B: before culture, C-D: after 2 days of culture without hormones and without sponge, E-F: after 2 days of culture with hormones (30 nmol/l estradiol +50 nmol/l progesterone) and without sponge, -J: after 2 days of culture with hormones (50 nmol estradiol +50 nmol/l estradiol +50 nmol/l progesterone) and without sponge, doi:10.1371()ournal.pone.0014497.g001

Role of TWEAK on the uNK cytotoxic receptor NKp46. We compared the presence of the uNK cytotoxic receptor, NKp46, in patients with an IL-18 over-expression (if compared to the one observed in fertile patients) and either a low or high mRNA TWEAK expression.

The NKp46 protein was mainly detectable in the huminal and glandular epithelium and within the stroma around the spiral arteries. The NKp46 immanostaining was stronger in patterns with IL-18 over-expression and simultaneously a low TWEAK expression (Figure 4A) if compared with patients with IL-18 and TWEAK over-expression (Figure 4B). No variation in staining

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localisation was observed. Presence of TWEAK seems to control the potential cytotoxic role of IL-18 on uNK cells, in case of overexpression.

Does TWEAK regulate IL-III or the opposite? We compared the expression of TWEAK and IL-III under different stimulation to determine if TWEAK regulate IL-18 expression or be regulated by this cytokine.

Stimulation of recombinant protein IL-10 or its airthody in the culture medium did not influence subsequent TWEAK expression if compared to the control condition. Only addition of ethanolate as known inhibitor of the Map Kinase pathway induced a

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Figure 2. Endometrium cells death revealed by FITC-Annexin V antibody. A-D: after 2 days of culture, E-H: after 5 days of culture. A.E: without sponge and without hormones, B.F: without sponge and with hormones (50 nmol/1 estuadiol +50 nmol/1 progesterone), C.G: with sponge and without hormones D.H: with sponge and with hormones (50 nmol/1 estuadiol +50 nmol/1 progesterone), doi:10.1371/journal.pone.0014497.g002

significant TWEAK mRNA decrease (p=0.01) (Figure 5A). Likewise, there was no variation of IL-18 expression after 24 h of stimulation with recombinant TWEAK or its antibody in the rulture supernatant. As for TWEAK, only addition of ethanolate produced an important IL-18 mRNA decrease (p=0.07) (Figure 5B).

TWEAK expression is not regulated by IL-18 and IL-18 has no effort on TWEAK mRNA. These results highlighted TWEAK and IL-18 expression are independent one from the other in human endometrium but are both regulated via a Map Kisase pathway.

TWEAK and uNK cells. In order to verify whether TWEAK could counterbalance IL-10 cytotoxic effect on uNK cells, we quantified, in different cubure conditions, the expression of two proteins: NKp-86, which mediates uNK cells cytotoxic effects and TGF-01. [Transforming Growth Factor-4]) which promote the angiogenic effect of uNK cells. We added in culture media an excess of IL-III recombinant protein in create an endometrial pro-inflammatory environment in all culture conditions. Excess of recombinant TWEAK protein or in antibody was introduced in the medium too, in the aim to modify IL-18 effects. Addition of TWEAK in the culture medium had no effect on NKp46 or on TGE-BI expression when compared to the IL-18 haad over-expressed modifiem. However, in TWEAK antibody supplemented culture medium, we observed a significant increase of both NKp46 (Figure 6A) and TGF-Blexpression (Figure 6B. Only an imbalance between IL-18 and TWEAK production has an impact on the two protein expressions.

Discussion

We demonstrated, using a collagen sponge gel support with hormonal substitution, that the endometrial cells are viable during

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four days, do not dedifferentiated and are able to produce endometrial "markers". Indeed, the cells conserved their morphology and functionality even with a minimal hormone concentration. Our model was very simple to use and we maintained interaction between the different cellular types (spithelial, stromal, endothelial and immunological relia). It seems to be a good system to study the production or expression of cytokines and growth factors during the window implantation in women. With this model we show that IL-18 and TWEAK expression in cadornetrium are independent our from the other but share the same way of regulation, the Map Kinase pathway. However, as previously suggested, we confirmed that TWEAK acts on the uNK cells cytotoxic receptor expression, by medifying one of the uNK cells cytotoxic receptor expression, NKp46.

The comprehensive functions of uNK cells still remain unclear. The two possibilities that are currently order investigation are that uNK, cells influence maternal mucosal and arterial function [22] and/or placental wophoblast invasion [23]. This assumption is further supported by the high levels of angiogenic growth factor (Vaccular Endothelial Growth Factor C, Placental Growth Factor, Augiopoletins 1 and 2, Transforming Growth Factor -[0] secreted by uNK cells from both non-pregnant endometrium and early decidua [24].

In human, Laird et al observed an increased density of CD 56^{bitden} (aNK cells) in mid hureal phase endometriam in women suffering from recurrent implantation failures compared with fertile controls [25] but did not evaluate either their cystoxic potential or cytokines content. As we previously reported that high IL-18/TWEAK redometrial mRNA expression correlated with a significant increase of uNK cells recruitment, we investigated the role of these two cytokines on uNK cells cytotoxicity. Manaster et al, reported that hoth proliferative and secretory phase uNK cells express NKp46 and NKG2D, but do not express NKp44 and NKp30, at the opposite of NK cells from deciding which express NKp44 and NKG2D, but NKp30, NKp44 and NKG2D [26]. We then decided to choose NKp46 as target to evaluate cytotoxicity of uNK cells in endometrium. Our immunohistochemistry and PCR results show that TWEAK doesn't act on IL-18 expression, but sremu to control IL-18 related cytotoxicity on uNK cells when IL-18 is overexpressed. Thus, TWEAK appears as a modulator to avoid endometrial uNK cytotoxicity induced by IL-18 over-expression.

Besides we reported that TWEAK also influence TGF-β1 cytokine production. The biology of the TGF-B family is complex and the various roles attributed to TGF-B in the uterus are still unclear. Our results revealed that when IL-18 is over expressed and TWEAK is inhibited by a specific neutralising antibody, an increase of TGF-\$1 is observed. We postulated that the observed TGF-B1 increase could reflect a retro-control loop; at TWEAK could not protect anymore endometrial cells from inflammation, others proteins with anti-inflammatory effect will be produced, like TGF-β. Erikason st al recruitly reports that endometrial production of TGF-B regulate uNK cell proliferation but also cytokine production and in particular the B/N-y production [27]. The combined endometrial IL-18 over-expression with a TWEAK inhibition increase cytotoxic receptors of uNK cells which produce manhely pro-inflammatory cytokines like DNY Local TGE-B are produced to commerbalance the cytotoxic activities.

In summary, the present study presents IL-10 and TWEAK as two independent proteins possibly acting in synergy to maintain the angiogenic/cyrotoxic balance related to uNE cells. Such data about the controlled on larger number of patients, Taken together, these results strengthen the idea that IL-18 on TWEAK mRNA expression should be evaluated as functional hiomarkers of an adequate/inadequate uttribe receptivity in order to detail the mechanisms of failures.

Material and Methods

Patients

All patients provided informed consent and this investigation was approved by our Institutional Review Board (CCPPRB), 30

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Figure 4. NKp46 immunostaining. Patients with an overexpression of IL-18 and with low (A) or high level of TWEAK (B). Magnification is 20X (first column) and 40X (second column), ep: epithelium, s: stroma, gl: glands, sa: spiral arteries. doi:10.1371/journal.pone.8014497.g004

women were enrolled in this protocol, 21 were Caucasian, 6 were from North of Africa and 3 from Africa. All subjects were involved in the Assisted Reproductive Technology program for investigations related to their infertility: 13 women experienced unexplained recurrent spontaneous abortion and 17 repeated and unexplained implantation failure following in sites fertilization (FVF). They have no other remarkable pathological profile, 6 patients in this group had given birth to healthy infants. The inclusion criteria included a normal hormonal reserve (FSH<10 mIU/ml) and patients were less than 38 years old (with a mean of 34,54).

8 patients were included to document NKp46 immussistaining: all patients had a high IL-18 mRNA expression and either a low (4 patiens) or a high (4 patients) TWEAK expression, 22 other



Figure 5. TWEAK (A) and IL-18 (B) mRINA expression. From endomstrial microhistoculture in 2 petients. The control modum was only supplemented with 50 nmol/L of homones (estradio) and progesterone). Ethanolate was added to the medium as an inhibitor of Map Kinase pathway. Estradioi and progesterone were added in all media. doi:10.1371/journal.pone:3014497.g005

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Figure 6. NKp46 (A) and TGF-6 (B) expression. From endometrial microhistoculture in 5 different patients. Recombinant human IL-18 (L-18) alone or with anti-human TWEAK (IL-18 + AC TW) were added to the medium. Esteadiol and progetterone were added in all media. doi:10.1371/journal.pone.0014497.g006

patients were included for the microhistoculture experiment. Individual analysis of each endometrial immune environment was detailed as previously described [28] and showed either a normal local uNK recruitment and activity or a cytokine–related uNK, depletion. None had a pro-inflammatory profile before the microhistoculture in this group.

Endometriam Biopsy, Prior the endometrial biopsy, a systematic endometrial ultrasonic examination was performed to record the endometrial volume, exclude any intra-sterine absormality of the uterine cavity and document the overall uterine vascularisation. As previously described [29,30,31], a single operator (N.L.) employed an automated 5–9 MHz transvaginal three-dimensional transducer (Voluson 730 expert, GE Medical System, France) to acquire power Doppler information from the uterus using identical settings and technique in all cases as follows: tissue harmonic imaging, mild; pulse repetition frequency, 0.6 khz; wall motion filter, low; gain, -7.8; high quality, rise 0.2. The volumetric and vascular measurements were undertaken using the Virtual Organ Computer-aided AnaLysis (VOCAL⁷³⁴, GE Kretz) imaging 4-D VIEW program with the 15° rotation step. This allows the user to define the volume of interest manually and applies a shell of variable thickness that parallels the originally defined unface contour, thus allowing delineation of the myometrial-endometrial border. Intensity and quantity of the power signal was defined in the sub-endometrial volume (endometrial volume +5 mm from the

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border). Endometrial biopsies were performed (without anesthesia) during a monitored natural cycle 7 to 9 days after the ovulation surge (LH) identified by serial blood tests. Biopsies were performed with a standard Cornier pipelle (CCD Laboratories, Paris, France). This method collects material from the superficial layer and not the basal one, minimizing variations due to sampling from different depths. One portion of the sample was embedded in paraffin for immunostaining, another one was immediately placed in a tube with RPMI 1640 Glutamax medium at 4°C for endometrium histoculture. The last portion transferred into RNA Stabilization Solution (RNA Later, QLAGEN, Courtaboued, France) was used to quantify the baal level of TWEAK and IL-18 in fresh endometrium by real time PCR.

Endometrial Micro-histoculture

5 to 9 patients were included in each experiment and each patient was her own control. The experiments were performed in triplicate. During the 2–3 hours following collection, endometrium biopsies were washed with RPMI 1640 medium. Collagen sponge gels (1.5 cm², Gelfnant[®], Pharmacia Upjohn, Kalamazoo, MI, USA) were placed into the welb of 12-well plates containing 1,5 ml per well of RPMI 1640 Glutamax medium supplemented with 15% heat-inactivated fetal calf serum (FBS), 1% penicillinstreptomycin, 1% non-essential amino acids, 1% sodium pyruvate (Gibco BRL, Life Technologies, France). Endometrium were cut into 1–2 mm blocks and placed on top of the collagen sponge gels at the interface between the medium and the air (3 blocks per collagen sponge and per well).

To validate the micro-histoculture model, four different conditions were realised with or without collages sponge gel and with or without 50 nmol/I of B-estradiol and progesterone (Sigma, France). Estradiol and progesterone were added to the medium every day. Micro-histocultures were maintained at 37°C (5% CO₂). For the experiments, we used the collagen sponge gel with 50 nmol/1 of B-estradiol and progesterone. Antibodies, recombinant protein or inhibitors were added to the medium after 24 hours of culture. Biopsies pieces were collected after 24 hours of incubation with the recombinant proteins, antibodies or inhibitors and transferred into RNA Stabilization Solution (RNA Later, QIAGEN, Courtaboueuf, France) to be stored at -80°C. The RNA extraction was performed several days later. Recombinant human TWEAK (75 ng/ml), anti-human TWEAK antibody (400 ng/ml), recombinant haman IL-18 (100 ng/ml) and anti-human IL-18 antibody (900 ng/ml) were from R&D sytems (Lille, France). Ethanolate (5 µmol/I), an inhibitor of MAP. Kinase pathway was purchased from Sigma (St Quentin-Fallavier, France).

Evaluation of cell death

The other part of micro-explants was placed into a well of 24 well plates with 1 ml of collagenase IV (1 mg/ml, Laboratoires Eurobio, Courtaboeuf, France) during 2 hours at 37°C. The dissociated cells were washed with PBS and incubated in a buffer containing 500 µl Hepes and 10 µl Annexin V-Pluos (Roche Diagnostics, Meylan, France) during 10 min at 37°C in darkness.

Cells were rinsed with Hepes and observed under a fluorescence microscope. Dapi (4',6-diamidine-2-phenyl indole) was used to stain nuclear cells (CML, Nensours, France).

Luminex

This experiment was realised using endometrial samples from 5 different patients. The culture medium was collected and fresh medium was added on days 2 and 3. Medium was frozen at -20°C until away by the Laminex Technology (Bio-Rad®). This rechnology was used to simultaneously read concentrations of IL-1β, IL-1RA, G-CSF, PDGF, 1P-10, MCP-1, MIP-1β and RANTES. The assay was performed according to the manufacturer's instructions.

Total RNA extraction and reverse transcription

Endometrial fresh tsiopsy and micro-explants were disrupted in the bysis buffer of a RNeasy kit (QIAGEN, Courtabeuf, France) using an Ultra Turrax T15 (IKA-WERKE). Total RNA was extracted using the RNeasy kit according to the manufacturer's instructions. Additional DNase digestion was performed during the extraction process (RNase-free DNase set, QIAGEN, Courtabeuf, France),RNA was smeed at ~80°C until use. Total RNA (1 µg) was reversed transcripted into cDNA using random primers and Superscript III (Invitrogen, Carhbad, CA) according to the manufacturer's instructions. Controls without reverse transcriptase were systematically performed to detect genomic DNA contamination. The cDNAs were stored at ~20°C until use.

Real Time PCR

Specific primer for TWEAK [32], IL-18 [5], TGF-J1 [24] and Ribosomal protein L13A (RPL13A) [53] were previously published. Primers used for NKp46 (NKp46-s: gactagagaggagcagga and NKp46-an augagettgtgtgttcageette) were designed using the Universal Probe Library Assay Design Center (www.rocheapplied-science.com) and sequences were searched against Gen-Bank sequences with the BLAST program to ensure the specificity of primers. Real-time PCR was carried out using a LightCycler 480 apparatus (Roche, Meylan, France). Reactions were set up using the following final concentrations: 0.5 µM of sense and astisens primers, 1X 400 SYBER Green master mix and 4 µl of 1/20 diluted cDNA. Cycling conditions were as follows: denaturation (95°C for 5 min), amplification and quantitation (95°C for 10 a, 60°C for 10 a and 72°C for 15 a) repeated 40 times, a melting curve program (65-95°C with a ramp rate of 2,2°C/4) and a cooling step to 4°C. In addition to the no-reverse transcription and no-template controls, an independently interrun calibrator (IRC) was included in each RT-PCR away. This IRC was obtained from Blast-cella for TWEAK, TGF-B and NKp46 and from placenta for IL-18, RPL-13.

In each away, an aliquot of the IRC cDNA was 20 times diluted and was submitted to the qPCR protocol as the unknown samples. PCR efficiencies for each quantified target and reference were calculated using known serial dilutions of each specific cDNA. Data were analysed using the LightCycler[®]480 Software release 1.5.0. Each specific target transcription level was normalized to the geometric mean of the transcription levels of the inference gene, using the Advanced Relative Quantification of the LightCycler[®]480 Software. Each gene specific amplification efficiency was specified. For each sample, results were expressed in concentration ratio (target/reference mRNAs).

Immunohistochemistry.

Micro-explaints (on days 1 and 5) were fixed in 4% buffered formaldehyde (96% Phasphate Buffer Saline or PBS) overnight at 4°C. They were washed with PBS, dehydrated in alcohol (70%, 95% and 100% ethanol) before being embedded in paraffin. Paraffin sections (5 µm thick on Superfrost glass slides) were deparaffined in histolernon and re-hydrated with alcohol. Endogeneous peroxydases and biotin activities were inhibited by incubation of the slides in a specific solution (Peroxydase Blocking solution, DAKO, Trappes, France and Avidin/Biotin Blocking Vector kit, Abcys, Paris, France) or by incubation with 0.3% hydrogen peroxide. Protein blocking was achieved by a 20 min

incubation in PBS (Phophate Buffer Saline) with 1% of home serum and 5% human normal serum (blocking serum).

✓ Morphological evaluation: Cytokeratin staining was made using mouse anti-human cytokeratin pan KL1, dilution 1/40 (Clinisciences, Montrouge, France) and vimentin staining by using mouse anti-human vimentin V9, dilution 1/200 (Biogenex, France), KL1and vimentin immunostaining detection was performed using the appropriated secondary antibody from kit Vectastain (Vector ABC, Abcys, Paris, France) revealed with 3.3" diaminobenzidine (DAB, DAKO, Trappes, France).

✓ NKp16 experiments: Physiological ranges of IL-18 and TWEAK mRNA expression have been previously established in control fertile women [28]. Briefly, TWEAK and IL-18 expression were normalised using geometric mean of three validated references genes (for the endometrium). We then calculated the mean (+/- SEM) of these ratio in our fertile patient group allowing us to obtain the normal range both for IL-18 (3.8 to 6) and TWEAK (4.2-6.5) inRNa expressions. We retrospectively selected endometrial samples with a high IL-10 mRNA expression (over 6.17) and low (below 4.1) or high TWEAK (over 6.86) mRNA expression to compare their NKp46 and TGF-B protein expression. NKp46 staining was made using a polyclonal goat antihuman NKp46 antibody, concentration 4 µg/ml, (R&D Systema, Lille, France). Between each steps the alides were rinsed three times with PBS. NKp46 immunostaining detection was performed using the appropriated secondary antibody from kit Vectatinain (Vector ABC, Abcys, Paris, France) for 20 min followed by incubation with a peroxydase-conjugated streptavidin for 30 minutes and then by use of substrate buffer supplemented with liquid DAB and hydrogen peroxide substrate solution (DAKO, Trappes, France).

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Counterstaining was performed with hematoxylin and slides were mounted with Eakitt medium for light microscopy observation. Negative controls were performed both by omission of the primary antibody and by its replacement by an appropriate isotype control. All slides were analysed with a fully automatic virtual slide scanner (DotSlide BX micoscope, Olympus, Hamburg, Germany).

Statistical analysis

Wilcoxon test was used to compare production of cytokines and growth factors (in different conditions of hormones concentration) and TWEAK, IL-18, NKp46 and TGF-B1 mRNA expression in each culture conditions. The statistical assessments were performed using the StatView software (Ahacus Concepts, Inc., CA, USA). Significance was set up at p≤0.05.

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Author Contributions

Conceived and designed the experiments: MP VS RW FdM GC JMF C. Munaut NL, Performed the experiments: MP MR SD C, Morvan C, Munaut, Analyzed the data: MP MR VS GC C. Munaut NL, Contributed reagents/materials/analysis tools: MP SD C. Manast. Wrote the paper: MP

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ORIGINAL PAPER

Specific and extensive endometrial deregulation is present before conception in IVF/ICSI repeated implantation failures (IF) or recurrent miscarriages

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Abstract

The objective was to examine if IVF/ICSI repeated implantation failures (IF) or recurrent miscarriages (RM) could be related to preconceptional endometrial deregulations. IF was defined as the absence of pregnancy despite the transfer of at least ten IVF/ICSI good quality embryos, and RM as having at least three unexplained miscarriages. Fertile controls (FC) were women who had given birth at least once. Endometrial biopsy was performed in the mild luteal phase of a non-conceptual cycle (five women were selected in each group), Affymetrix chips (GeneChip Human Genome U133 Plus2.0 Array) were used for hybridization. Data were normalized by the gcRMA method, and raw p values adjusted by the Bonferroni procedure (196). Differential expression of selected genes was analysed using real-time PCR. Gene networks and biological functions were explored using the Ingenuity Pathways Analysis software. Endometrial gene expression profiles at the time of uterine receptivity differ dramatically in the endometrium among FC, RM, and IF patients. Compared to FC, 2126 and 2477 genes are differentially expressed in IF and RM groups, respectively, and 2363 between IF and RM. In both conditions, differential gene expression referred mainly to DNA transcription and expression. Other main cellular functions deregulated in IF conditions correspond to cell morphology, cellular development, cell cycle, and cellular assembly, while in RM conditions, deregulated cellular functions relate to cell signalling (degradation of cyclic AMP and calcium metabolism) and cellular maintenance. In both conditions, there is an over-representation of deregulations related to the haematological system. In the IF condition, cell-mediated immune response and nervous system development and function are highly deregulated, while in RM patients, main deregulations are in organ and tissue development, ane response, and muscular system development and function. Extensive endometrial deregulations humoral imm are present before conception in patients who experienced IF or RM with both distinct and common deregulation. Copyright © 2011 Pathological Society of Great Britain and Ireland. Published by John Wiley & Sons. Ltd.

Keywords: microarray; endometrium; uterine receptivity; IVF/ICSI; recurrent miscarriages; implantation failure; infertility

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No conflicts of interest were declared.

ORIGINAL PAPER

Specific and extensive endometrial deregulation is present before conception in IVF/ICSI repeated implantation failures (IF) or recurrent miscarriages

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Abstract

The objective was to examine if IVF/ICSI repeated implantation failures (IF) or recurrent miscarriages (RM) could be related to preconceptional endometrial deregulations. IF was defined as the absence of pregnancy despite the transfer of at least ten IVF/ICSI good quality embryos, and RM as having at least three unexplained miscarriages. Fertile controls (FC) were women who had given birth at least once. Endometrial biopsy was performed in the mild luteal phase of a non-conceptual cycle (five women were selected in each group), Affymetrix chips (GeneChip Human Genome U133 Plus2.0 Aeray) were used for hybridization. Data were normalized by the gcRMA method. and raw p values adjusted by the Bonferroni procedure (1%). Differential expression of selected genes was analysed using real-time PCR. Gene networks and biological functions were explored using the Ingenuity Pathways Analysis software. Endometrial gene expression profiles at the time of uterine receptivity differ dramatically in the endometrium among FC, RM, and IF patients. Compared to FC, 2126 and 2477 genes are differentially expressed in IF and RM groups, respectively, and 2363 between IF and RM. In both conditions, differential gene expression referred mainly to DNA transcription and expression. Other main cellular functions deregulated in IF conditions correspond to cell morphology, cellular development, cell cycle, and cellular assembly, while in RM conditions, deregulated cellular functions relate to cell signalling (degradation of cyclic AMP and calcium metabolism) and cellular maintenance. In both conditions, there is an over-representation of deregulations related to the haematological system. In the IF condition, cell-mediated immune response and nervous system development and function are highly deregulated, while in RM patients, main deregulations are in organ and tissue development, humoral immune response, and muscular system development and function. Extensive endometrial deregulations are present before conception in patients who experienced IF or RM with both distinct and common deregulation. Copyright @ 2011 Pathological Society of Great Britain and Ireland. Published by John Wiley & Sone, Ltd.

Keywords: microarray; endometrium; uterine receptivity; IMF/ICSI; recurrent miscarriages; implantation failure; infertility

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No conflicts of interest were declared.

Introduction

The endometrium is remodelled throughout the menstrual cycle and exhibits only a short period of receptivity in the mild luteal phase, known as the 'implantation window', which is crucial for both implantation and gestation [1]. The endometrium becomes receptive to blastocyst implantation 6–8 days after ovulation and remains receptive for 4 days (cycle days 20–24) [2]. Improving our knowledge in this area may be critical to understanding the endometrial physiology. In humans, this phenomenon of differentiation of the maternal compartment regulates the implantation process and is independent of the presence of

Copyright © 2011 Pathological Society of Great Britain and Ireland. Published by John Wiley & Sons, Ltd. www.pathsoc.org.uk the embryo (in contrast to rodents) [3]. We postulated that preconceptional endometrial expression may influence communication in the conceptus maternal environment and could account for the subsequent reproductive outcome. We therefore decided to examine patients with a history of repeated implantation failures or recurrent spontaneous abortion to determine whether earlier endometrial deregulation is detectable before conception. Using a large-scale microarray analysis, we compared endometrial gene expression at the time of the implantation window in fertile control patients with women displaying previous IVF/ICSI repeated implantation failures or recurrent unexplained miscarriages.

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Materials and methods

Patients

Three groups of patients were included in the analysis: patients with a history of IVF/ICSI implantation failures (IFs); patients with a history of unexplained recurrent miscarriages (RMs); and fertile control patients (FCs). We selected subjects in two steps, five subjects representing each category to reduce variations within each group and increase consistency in data collection (Table 1). All subjects were less than 38 years old and mild luteal phase was demonstrated by the endometrial biopsy according to Noyes' criteria [4] (Table 2). Adequate quantity and quality of endometrial RNA should be available for microarray hybridizations.

Fertile patients (n = 15) who successfully delivered after the first or second attempt of either IUI or IVF/ICSI related to a male infertility were included. They were re-evaluated before replacement of frozen embryos or a new cycle of IUI. Three-dimensional (3D) ultrasonography documented a normal endometrial volume. Ovarian reserve [FSH, antral follicle count (AFC)] was normal. Absence of miscarriage was selected as a secondary criterion.

In the IF group, patients (n = 30) with repeated, unexplained failure of IVF-ET (defined as absence of pregnancy despite the transfer of at least ten embryos over several cycles) were included. Patients with a low endometrial volume (<2 cm) or the presence of sub-mucous fibroma or polyps were excluded. As secondary criteria, all embryos transferred had a fragmentation rate of less than 20% and were at least at the four-cell stage by day 2 after IVF. A good hormonal reserve (FSH <10 mIU/ml, AFC >9) and normal responses to ovarian stimulation were also included as additional selection criteria. Azoospermia and severe oligo-teratospermia were exclusion criteria.

Recurrent miscarriages (RMs) or pregnancy losses (RPLs) (n = 25) were defined by at least three pregnancy losses between 6 and 12 weeks of gestation unexplained by autoimmune (anti-phospholipid antibodies as lupus anticoagulant and anti-cardiolipin antibodies), thrombophilic, and genetic (protein S, protein C, Factor XII, Factor XIII, Factor V Leiden mutations, Factor II G20210A, MTHFR, homocysteine, parental karyotype) explorations. As secondary inclusion criteria, a normal ovarian reserve (FSH <10 IU, AFC \sim 9) and normal sperm with normal DNA (ragmentation (below 13%) were required. Patients with a thin endometrium (<2 cm³), synechia or uterine malformation were excluded.

Endometrial evaluation in the middle luteal phase

Endometrial evaluation combining ultrasonographic evaluation and endometrial biopsy was performed during a monitored natural cycle 7–9 days after ovulation as described previously [5]. All patients gave fully informed consent for the research, and the Institutional Review Board approved this investigation (CPPRB, protocol 01–78).

Biopsies were performed with a standard Pipelle de Cornier[®] (CCD Laboratories, Paris, France). One portion of the sample was embedded in parafilin for histological dating and the rest was transferred into RNA Stabilization Solution (RNA Later) and stored at -80°C.

	Fertile controls (FEs)	Inglastation failure (IF)	Recurrent miscarriages (RMs)
Selection Primary orberta	15 Agt <38 years Successful delivery Normal 30 endometrial US Lutesl shase	30 Age <39 years >10 aaad embruos transferred	25 Age <38 years >3 RMs over 6 and 12 weeks
Secondary cilieria	No miscarriage	Normal ovarian reserve Normal ovarian response to stimulation Optimal development of transferred embryos	Normal ovarian reserve Normal spenn No synechia
RNA	Appropriate quantity and quality of SNA	Eaclusion of accespennia and severe ADT Appropriate quantity and quality of RNA	Normal endometrial thickness Appropriate quantity and quality of RNA
	6	5 ·	1 C

Table 2. Patient characteristics

	Fertile controls (FQ)	Implantation failure (IF)	Reservent miscarriages (RMs)
Mean age (min-max), years Mean BMI (min-max)	23 (29-37) 23.8 (21-27.9)	31.3 (28-35) 22.6 (19-29)	31 (25-36) 20.6 (18-25)
Primary or secondary infertility	All secondary male infertility First leith after the first or second attempt of IUI (2), or ICS (2)	A primary intertaility 1 secondary intertaility (1 HW)	All secondary infertility
Tebacco	None	None	None
Endometrial volume (day blogsy) cm ³	3.072	3.38	3.34
Mean sterine artery index of pairatility	9.94	143	2.45

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Table 1. Sample selection process

Gene symbol	Gene description	Firm	Catalogue No
TYMP	Thymidine photohorylate	Giagen	GT00238683
CDen.	Chan exclerible, INF receptor superfamily member 5.	Olagen	0001128699
CSFIR	Colony stimulating factor 3 receptor (granulocyte)	Qiagen	0100095522
CYPSAA	Cytochrome P450, family 2, subfamily A, polypeptide 4	Disgen	QT016/2608
HAHA	Retinuic acid receptor alpha	Qiapen	GT0c095865
10051.0	Inducible T-cell co-stimulator ligand	Qiagen	@focoo4eee
11.18	Interievkin 18 Sinterferon-gamma-inducing factor)	Qiagent	0100014560
PLANE	Plasminogen activator, urokinase receptor	Giagen	0700076447
CD40141	CD40 ligand	SABibidiences	FPH002138E
BCL2L10	BCu2-like 10 (apoptosis facilitator)	SAElosciences	7PH15924A
F00P3	Forshread box Pa	SABIISCHITUS	PPHOLOGOUR
82M	#-HICTUTCICCATUTTICATIGTATCT-3	Europentec	1100000
	S'-TCTCTBCTCCCCACCTCTAAET-3'	a nave we have a	
8PL13-A	5'-CCTGGAGGAGAGAGAGGAAGAGA-3'	Eurogenter:	1.00
	8"-TEGAGGACCTICTOTOTATTIOTCAA-9"		
THP:	17-TOCACADDAGCCAADADTGAA-3"	Turopette:	2

Table 3. Primer references for RT_nPCR

Endometrial RNA extraction - quality control and cDNA synthesis

V-CACANCACADETECCECACCA-Y

Real-time quantitative PCR (RT-gPCR)

Total RNA was isolated with the Qiagen RNeasy mini kit (Qiagen, Courtabeuf, France) including the RNase-Free DNAse set. Recombinant placental RNase inhibitor (10 units/µl of extracted RNA) was added to prevent RNA degradation. RNA quantity and quality were confirmed by the analysis with an Experion system and RNA StdSens analysis kit (Bio-rad, Marnes la Coquette, France). RNA was stored at -80°C. Total RNA (1 µg) was first reverse-transcribed into cDNA using random primers and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

Transcriptomic analysis

Four micrograms of total RNA was labelled using the GeneChip" Expression 3' Amplification One-Cycle Target Labeling kit (Affymetrix, Santa Clara, CA, USA) following the manufacturer's protocol. The cRNA was hybridized to the Genechip Human Genome U133 Plus 2.0 Array (Affymetrix). Briefly, doublestranded cDNA was synthesized from 4 µg of total RNA primed with a poly-(dT)-T7 oligonucleotide. The cDNA was used in an in vitro transcription reaction (IVT) in the presence of T7 RNA polymerase and biotin-labelled modified nucleotides for 16 h at 37 °C. Biotinylated cRNA was purified and then fragmented (35-200 nucleotides) together with hybridization controls and hybridized to the microarrays for 16 h at 45°C. Using Fluidics Station (Affymetrix), the hybridized biotin-labelled cRNA was revealed by successive reactions with streptavidin R - phycoerythrin conjugate, biotinylated anti-strepatvidin antibody, and streptavidin R-phycoerythrin conjugate. The arrays were finally scanned with an Affymetrix/Hewlett-Packard GeneChip Scanner 3000 7G.

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The differential expression of selected genes was confirmed by RT-qPCR using LightCycler[#] 480 Multiwell Plates 96 (Roche Diagnostics, Basel, Switzerland), LightCycler 480 SYBER Green I master, and primer sets defined in Table 3. Each reaction was carried out in duplicate using 4 µl of diluted cDNA (20 times, 2.5 ng/ml) and 500 nM of each specific primer in a 10 µl final reaction volume. No-template controls, no-reverse transcription, and an inter-run calibrator (IRC) were included in each assay. Three reference endogenous genes (TBP, RPL13A, and 82microglobalin) were used in endometrial samples [6]. Data were analysed using LightCycler[®] 480 Software release 1.5.0. Each specific target transcription level was normalized to the geometric mean of the three reference genes using Advanced Relative Quantification of the LightCycler 480 Software. Specific amplification efficiency for each gene was specified and expressed as a concentration ratio (target/three reference mRNAs).

Data analysis

Data obtained after Affymetrix microarray hybridization analysis were imported into the R environment for statistical computing (http://www.r-project.org) and normalized with the germa algorithm [7]. Differential analysis was performed with the 'anapuce' package of R2 (http://www.agroparistech.fr/mia/doku.php?id== productions : logiciels). A double-sided, unpaired t-test was computed for each gene hetween the two conditions. Variance of the difference in gene expression was split between subgroups of genes with homogeneous variance [8]. The raw p values were adjusted by the Bonferroni method, which controls the familywise error rate (FWER). A gene was declared differentially expressed if the Bonferroni-corrected p value was less than 0.01. In a second step analysis following the validation by RT-qPCR, we restricted

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the analysis to all genes with a significant difference (delta) over 0.3 (log 2) in order to reduce the number of genes to be analysed. Gene networks and biological functions were determined using Ingenuity Pathways Analysis software (IPA, Ingenuity Systems, http://www.ingenuity.com).

CEL files have been published as GEO file number GSE26787.

Results

RT-qPCR validation and comparison of differentially expressed genes (DEGs) among FCs, IFs, and RMs

Endometrial gene expression profiles at the time of uterine receptivity differed dramatically in the endometrium antong the FC, IF, and RM subjects. Applying the Bonferroni-corrected *p* value below 0.01 alone, only 727 of the 7706 DEGs were found to be common between fertile, IF, and RM. Compared with FC, 4519 genes were found to be differentially expressed in the IF group and 4204 in the RM group. From 4519 genes between the FC and IF groups, 2540 differentially expressed genes were specific to IF, while 1979 were also found to be differentially expressed in RM conditions, compared with FC (Figure 1A).

In an attempt to reduce the number of genes to be analysed, we used the RT-qPCR efficiency to define the threshold of differences that would not allow us to confirm differences observed in the microarray as significant in RT-qPCR (Table 4). It appears that we were able to confirm significant differences (p > 0.05) using RT-qPCR for all the genes tested having a difference over 1.35-fold in the microarray study, while differences below that were insignificant except for one gene, *RARA* (Table 4).

In such a strict selection, 699 genes out of 3877 differentially expressed genes were still common to the three conditions. Compared with FC, 2126 genes were found in the IF group and 2477 in the RM group. From 2126 differentially expressed genes between the FC and IF groups, 877 (410 + 467) were specific to the IF condition. In comparison to FC, 1249 (699 + 550) were also found to be differentially expressed in RM conditions, compared with controls (Figure 1B). Of the 2477 differentially expressed genes in the RM conditions versus controls, 1228 DEGs were specific to recurrent miscarriages.

Furthermore, 2363 genes were found to be differentially regulated between IF and RM, among which 1166 were reported to be differentially expressed in IF versus fertile conditions and 1373 in fertile versus RM conditions. The Venn diagram in Figure 1 summarizes the overall categorization of differential expression between the three conditions.

Distinct biological functions are expressed in the endometrium in patients displaying previous IF and RM as compared to FC

Genes were clustered for network of defined categories as (1) molecular and cellular functions and (2) physiological system development and function (Table 5) in the three groups. Details of genes differentially expressed for some specific categories are listed in the Supporting information, Appendix. In both IF and RM conditions, gene expression was substantially affected [190 transcripts in IF (Supporting information, Appendix, list 1) and 198 in RM (Supporting information, Appendix, list 2)], especially the transcription and expression of DNA.

Main molecular and cellular functions differentially and specifically affected between IF and fertile relate to cell morphology (108 transcripts, Supporting infor-mation, Appendix, list 3), cellular development (196 transcripts, Supporting information, Appendix, list 4), cell cycle (59 transcripts), and cellular assembly and organization (107 transcripts). Specifically, in IF conditions, the morphology of blood cells (15 transcripts) and normal cells (28 transcripts) as well as their morphogenesis (48 transcripts) and shape changes (52 transcripts) were substantially altered (details in Support-ing information, Appendix, list 3). Differentiation (132 transcripts), maturation (32 transcripts), and developmental process (124 transcripts) mainly affected blood cells, leukocytes, and lymphocytes. This was especially true in the cases of T lymphocytes and stem cells in IF endometrium (Supporting information, Appendix, list 4).



Figure 1. Vern diagram according to the initial condition: fertile (F), implantation failure (IF), and recurrent miscarriage (RM). (A) Dysregulated gene with a Bonferroni-corrected p value over 0.01. (B) Dysregulated gene with a Bonferroni p value over 0.01 and a delta of difference over 1.35 (0.3 in log 2).

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Table 4. Validation of the experiment by RT-qPCR

	Fold express	Ion FAF		Fold expression F/RM		
Target gene	Micro-array	HT-PCR	p value*	Micro-array	81-908	p value*
70-FC0F	48	5.54	0.02		5.54	0.02
actust an	25	19.7	0.04	NS	-	0.22
CSF-3R	12	3.34	0.02	NS	-	0.0
7CIDSUF	11.8	2,27	0.04	NS		0.62
PLAUR	7	2.36	0.02	NS	-	0.08
RARA	2	0.6*	35	NS.	-	0,08
12-38	4.85	1.64	0.02	NS.		0.62
CD40	2.00	3.27	0.04	1.73	3.44	0.01
CD4010	1.2.0	-	0.14	1.34	2.13	0.05
CYP2A4	1.37		0.72	NS		0.67
FOXP3	1.09	-	0.32	0.58	0,68	0.03

^{*}Kruskal-Wallis test.

NS : Non significant

Table 5. Distinct biological functions differentially expressed in patients displaying previous IF and RM compared with control endometrial conditions

Groups	Molecular and Cellular Functions	No of genes	Physiological System Development and Function	No of genes
IF versus FC	Cell morphology	10E	Haematological system development and function	138
	GENE CAUTEDAUN	100	Raematopolesio	86
	Cril development	196	Lymphoid Tissue structure and development	18
	Cell cycle	石田		
	Cellular assembly and organization	307	Nervous system development and function	26
			Celi-mediated immune response	8.9
HM versus	Gene expression	196	Organ development	125
FC	Cell signating	51	Tasue development	85
	Cellular function and maintenance	26	Haematological system development and function	123
	Nucleic acid metabolism	34	Humoral immune response	22
	Small molecule biochemistry	68	Skeletal and muscular system development and function	87

In RM versus fertile conditions, molecular and cellular deregulation was present in cell signalling (51 transcripts), cellular function and maintenance (96 transcripts), nucleic acid metabolism (14 transcripts), and small molecule biochemistry (68 transcripts). In RM conditions, cell signalling was mainly affected by the inhibition and degradation of cyclic AMP, as well as abnormal metabolism of calcium and abnormalities of the formation and assembly protein-protein complexes. Cellular function and maintenance mainly concerned lymphocyte homeostasis, T-cell development, and differentiation of T cells.

In the 'physiological system development and function' category, haematological system development and function was the common deregulated category for the IF and RM groups [138 transcripts in IF—list 9 (Supporting information, Appendix) and 123 transcripts in RM—list 10 (Supporting information, Appendix)]. In both IF and RM conditions compared with FC, abnormal functions were related to haematopoiesin (76 and 90 transcripts), differentiation of leukocytes (59 and 72 transcripts), lymphocytes (56 and 70 transcripts), especially T lymphocytes (54 and 65 transcripts), and differentiation of blood cells (59 and 74 transcripts).

In IF patients compared with FC, cell-mediated immune response (59 transcripts) and nervous system development and function (96 transcripts) were the top functions that were not regulated properly. In these IF patients, 'cell-mediated immune response' again reflected the abnormal T-cell development (mainly Th-2 differentiation and development) as well as the NK cell migration and transmigration of Th-1 cells. In IF conditions, 'the nervous system function' underexpression was related to neurogenesis (46 transcripts), and development (28 transcripts) and growth (36 transcripts) of neurites.

Main physiological abnormalities specific to the RM group were related to organ (125 transcripts) and tissue (85 transcripts) development, humoral immune response (22 transcripts), and skeletal and muscular system development and function (87 transcripts). In recurrent miscarriage conditions, the 'organ development' category referred to organogenesis (93 transcripts) and development of organs (78 transcripts). The tissue development category almost exclusively referred to the abnormal developmental process of muscle (42 transcripts). The 'skeletal and muscular system function tissue morphology' categories highlighted problems connected to the contraction of smooth muscle (27 transcripts). Genes found to be the most representative of differential expression between the three conditions are summarized in Table 6 (A-IF versus FC; B-RM versus FC).

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Not validated.

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Table 6. List of the top genes that are over- and under-expressed in IF (A) and RM (B) compared with FC

and and the second second second	(A) Top genes theregulated in IF versus FC		
8	Gene title	Gene symbol	
Underexpressed	Endothelial cell growth factor 1 (platelet-derived)	ECOF1	
	Ataxin 3-binding protein	A2BP1	
	Naked cylicity homolog (Drowphild)	NKD2	
	Beta-1,4 - N-acetyl-galactosaminyl transferase 3	B4GALNTI	
	BC12-like 10 (apoptosis facilitator)	BCL2110	
	Chioride channel 6	CLCNE	
	F-box grotein to	FEXIDIO	
	Cytochrome P4LO, family 3, subfamily A, polypeptide 4	CYP3A4	
	Transmembrane protease, serine 2	TMPRSS2	
	Unc-5 homolog C (C elegans)-like	UNCED	
	Two pore segment charitel 3	TPCN3	
	Colony-stimulating factor 3 receptor (granulocyte)	CORD	
	Sergin peptidase inhibitor, clade B (oxalbumin), member E	SERPINER	
	NADH dehydrogenase (utriquinone) 1 alota subcimplex, 11, 14,7 kDa	NDUFATT	
	Inducible T-cell co-atlevulator ligand	10051.0	
Overexpressed	Sterol-C4-methyl oxidase-like	SC4MOL	
	Hypothetical protein LOC263294	LOC253264	
	6-Hydroxytryptamine (serotunin) receptor 6	HTRO	
	Sanaptic Ras (CPuse activating protein 1 homotog (rul)	SINGAPI	
	Leucine-rich repeat containing 37, member A3	LARCENAE	
	Protein interacting with PRKCA1	PICK1	
	Immunoplobulin kappa variable 10-13	IGKV10-13	
	Dystrobrevit, beta	DINE	
	Effector cell poptidase receptor 1	EPR1	
	Peptidvl arginine delminase, type i	PACET	
	Adresomedullin 2	ADM2	
	X-profet aminopeptidase (aminopeptidase P) 3, putative	XD4UPEP1	
	Elastin (supravalvular aortic stenesis, Williams-Beuren sendrome)	ELN	
	KIAA1909 protein	KIAA1909	
	Kazal-type serine peptidase inhibitor domain 1	KAZALD1	
	(B) Too order demonstration BM wrong EF		
	Gene bitle	Gene symbol	
Underenteenteel	Naked extirle homoios (Departmin)	NUTR	
And a state of the	Atroin 1 Aladian nertain	ASCES	
	Melastorio cell affrecise melacule	MCAN	
	the Abarolog C IC elegand Jac	UNCHE	
	TIDA' TOD simulate perturns security like IS considered	TUR	
	WINEY function manifest &	WITCHA	
	COunt molecule immunosicibulin, according tetra	CDmil	
	Call annually remaining with TZ doub downin 1	COBIES	
	Carl growth regional with private storage i	LOC-PRIME	
	Projektion and acceptible former is the barrier of	000203040	
	entotnesar en growth ractor i Brazer-ternatul	AMERICAN INC.	
	Villavilla erreine	KIASTOCI	
	Termineter factor 3.07.8 immunication in antioners binding factors	100a	
	interdent factor 3 (EVA intervingenous) entance: smeling factor)	PLF3	
	their ninger protein also	1.747-2.52	
Overexpressent	Nucleoporin 210 kūs	NUP210	
	Aguagorin 3	AQP9	
	Growth arrest-specific 2	GAS2	
	Interleukin 1 mceptur antagonist	IL SRN	
	Gentrin-releasing peptide	GRP	
	Chromosome 1 open reading frame 113	Ctarf113	
	ILA domain family member 3	TEADD	
	Elastie	ELN	
	Atenin	ARTN	
	Synaptic Ras ETPice activiting protein 1 homolog (rat)	SYNGAP1	
	Similar to CE18125-PA	LOC730112	
	Carebally 4 reprinter	PTEL NO.	
	Chicolini + bischicol	Cartain	
	Splitting further a	SE4	
	Spring factor a BA/1-associated protein 3	GE4 BAIAPE	

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Figure 2. Illustration of the intricate dysregulation between immune deregulation, integrin pattern, and coagulation disorders in IF patients. Genes highlighted in green are underexpressed in IF patients compared to FC, and genes highlighted in red are overexpressed compared with FC.

Construction of related pathways leading to differential expression in IF conditions

Discussion

From the top differentially expressed genes and functions differentially expressed between the two conditions, we were able to correlate some of the differentially expressed genes to our context of uterine receptivity in the context of IF. Down-regulation of the expression of integrin patterns (ITGA6, ITGB3, ITGAL, ITGAL2B) appears to be connected to coagulation disorders (F2R, PLAUR or u-PAR, F12). Such canonical pathways are involved in T-cell differentiation (ICOSLG, CD40, IL-18, CD86) and may have an impact on the communication between dendritic cells and uterine natural killer cells (integrins, CSF-2, IL-18, CD40, CD86), as represented in Figure 2. Such down-regulation of the expression of interleukin-18 implicated in T-helper cell differentiation (Figure 3) and vascular remodelling seems also to relate to abnormal signalling of the colony-stimulating factor family expression (CSF-2 or GM-CSF and its receptor CSF-3R*) as well as abnormal chemokine expression (CxCR3) (Figure 4A). To illustrate some differences between IF and RM patients, no downregulation of the expression of IL-18 and CSF-3R was observed in the context of recurrent miscarriages (Figure 4B).

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A wide range of differentially expressed genes were detected in preconceptional endometrium as a function of previous history of either fertility or infertility. Such an influence of matricial preconceptional expression has been suggested by previous authors in animal models [9]. The main hypothesis is that both intertwined and coordinated endometrial and embryonic adequate expression is required for a normal implantation and gestation. Through a strict experimental design, we reduced as many intra-group variations as possible to increase the accuracy of our statistical analysis [10]. The number of differentially expressed genes reveals the extent of changes within the endometrium prior to conception in subjects who had experienced IF or RM. The challenge is to make sense of the vast amount of data generated while relative low fold changes are observed. Looking at gene expression in whole endometrial tissue may represent a limitation, as it may mask a higher level of changes in the different tissue components. We thus decided to analyse our data by observing cell functions regulated by the differentially regulated genes.

Previous human microarrays from endometrial biopsies focusing on uterine receptivity compared genomic expression in natural cycles at various stages of the menstrual cycle [11–15]. In addition, they compared

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Preconceptional endometrial environment affects implantation in humans



GRAPE CHIEF, ISBAN, HOVE, ILLER, MICHIN, MINIC MILLER, PERMIT, PRIMITER, MICHIN, MILLER, MINICI

Figure 3. Differentiation of T cells in IF and RM patients compared with FC.



Figure 4. Illustration of the differences between IF and RM for clue genes such as IL-1II and CSF-3R.

natural and hyperstimulated endometrial conditions [15,16] or evaluated the impact of endometriosis [17] or inert intrauterine device [18]. The design of our study was completely different since our aim was not to define uterine receptivity, but to gain new insights into the mechanisms and molecular pathways involved in inadequate uterine receptivity.

The analysis of molecular pathways suggests similarities but also key differences between IF and RM. The main similarities of differentially expressed genes between IF and RM relate to immune and haematological system development abnormalities, es-

Copyright © 2011 Pathological Society of Great Britain and Ireland. Published by John Wiley & Sons, Ltd. www.pathuoc.org.uk pecially deregulation of the differentiation and development of T lymphocytes and blood cells. Such immune disorders have been widely documented and have been examined by the evolving Th-1/Th-2/ Th-17 paradigm [19,20]. The implication of T regulatory cells as well as dendritic cells [21] in complement to the well-characterized mobilization of uNK cells at the time of implantation seems inadequate in both conditions. The high number of differentially expressed genes implicated in both the expression and the transcription of DNA shows the extent of the preconceptional dysregulation.

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A high incidence of uterine vascular abnormalities has been widely reported in such a context of failure [22,23]. Interestingly, the platelet-derived endothelial cellular growth factor (PD-ECGF) is represented in both situations in the top list of genes that are differentially regulated. PD-ECGF has been extensively studied over the past 15 years in the endometrium as one of the main sex-steroid-dependent pathways regulating endometrial angiogenesis with the VEGF signalling pathway [24,25]. However, high-throughput studies were carried out almost exclusively in the context of endometrial cancer, even when a previous study suggested its role in physiological endometrial decidualization [26].

We also observed substantial differences in gene regulation between IF and RM. While in both cases cellular functions were considerably affected, in IF the morphology, development, and organization of cells were the main targets, whereas in RM conditions cellular signalling and metabolism were found to be mainly defective. Such differences may suggest that the initial dialogue between embryonic and endometrial cells may be affected in IF patients, while it is present but deregulated for further placentation in RM patients,

Indeed, in IF conditions, we were surprised by the number of genes that were differentially regulated in relation to neuronal functions. Nerve fibres have recently been described to be present in excess within the endometrium in some cases of endometriosis [27]. None of our natients in the IF group had endometriosis in their past history, but for some authors, the presence of nerve fibres was considered a sign of local pain [28]. The dendritic cell-related pathway was also affected as were related molecules such as galectin-3 in the IP context, while several authors demonstrated their importance in establishing an environment of immune tolerance for pregnancy [29]. In RM, compared with IF conditions, a substantial difference was observed in the number of muscle genes that were differentially regulated. In addition, their contraction-relaxation process and molecular mechanism related to the transport of calcium and cyclic AMP were affected. Organogenesis and the development of organs were also affected. The importance of communication between the myometrium and endometrium, as well as the uterine junction zone, has been established for women undergoing miscarriage and pre-eclampsia [30].

Analyses of mild-luteal endometrial expression also suggest that early cross-talk takes place in order to allow the implantation process to occur. Studies performed in bovine models were able to demonstrate that endometrial expression shows drastic gene modifications related to the way that the embryo was produced in *in vivo* fertilization, *in vitro* maturation or cloning [31,32]. Mansouri-Attia *et al* raised the hypothesis that the endometrium could be a natural biosensor for the quality of the embryo. In human species, gene expression in decidualizing endometrial stromal cells showed significant variations whether the blastocyst was still in development or not [31]. Such data obtained in vitro suggest that the preconceptional state of the endometrium may impact the subsequent dialogue with the embryo.

The preconceptional impact of ovarian expression on the subsequent endometrial expression may also be raised. We previously described in individual follicular fluids that G-CSF (or CSF-3) was a noninvasive biomarker of oocyte competence for implantation [34-36], while GM-CSF was described as being essential in seminal plasma to stimulate local immune tolerance [37]. Subsequently, Scarpellini and Sbracia recently described in a randomized G-CSF versus placebo study that G-CSF was effective in increasing the ongoing pregnancy rate in RM patients [38]. In our microarray study, the G-CSF receptor gene was found to be differentially expressed and was listed in the top list and related to immune disorders as represented in Figure 4. As another example, BCL2L10, a protein involved as a pro-apoptotic regulator, is downregulated in IF conditions, while the same molecule has been described as an oucyte and embryo survival factor [39]. As the endometrium is now thought to be a biosensor for the quality of the embryo, such differential expression may have direct consequences on the initial embryo-uterus dialogue.

To conclude, this high-throughput analysis focusing on inadequate uterine receptivity suggests that preconceptional extensive endometrial deregulation is present in patients who have experienced IF or RM. Such differential expression may reflect the impaired ability of the endometrium to respond correctly to the embryo. Evidence of distinct and common pathways of differential regulation has been suggested and need to be further investigated using *ex vivo* models. The major remaining question is the identification of the maternal mechanisms that lead to such endometrial differential gene expression. Once identified, these pathways may be useful by providing new insights into targeted treatment design to prevent recurrent failures.

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Author contribution statement

NL, OS, JMF, and GC conceived the study. NL, CM, VS, and MR carried out the experiments. JA performed statistical analysis of the raw data. NL, CM, and OS analysed the data, and NL wrote the manuscript. All authors had final approval of the submitted versions.

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SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article.

Appendix. Details of genes differentially expressed for some specific categories (lists 1-6).

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7.3. Reference Article for the Pro Abortive Murine Model

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Seminal plasma peptides may determine maternal immune response that alters success or failure of pregnancy in the abortion-prone CBAxDBA/2 model

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ABSTRACT

Spontaneous abortion (resorption) in the DBA/2-mated CBA/J mouse involves a deficiency in Treg cell activity against paternal antigens at the time of mating. Preimmunization of female CBA/] by BALB/c splenocytes, but not DBA/2 splenocytes, protects against subsequent abortions after a CBAxDBA/2 mating. Previous immunogenetic studies with BALB/cxDBA/2 recombinants have indicated that H-2^d-restricted presentation of a single minor non-H-2⁴ peptide might be responsible for protection, while the product of a second independent allele might promote abortions. Using brefeldin-treated BALB/c and DBA/2 splenocytes, we found that incubation in BALB/c seminal plasma rendered DBA/2 splenocytes protective and DBA/2 seminal plasma eliminated protection. The active protective molety was <10 kD consistent with a peptide. DBA/2 seminal plasma contained a <10-kD peptide that boosted the abortion rate. Maternal H-2* CBA/J splenocytes were unable to present the protective activity. Amicon fractionation also unmasked a <10-kD activity in DBA/2 seminal plasma that could boost abortion rates when presented by RALB/c splenocytes. SELDI-TOF mass spectrometry proteomic analysis of <10-kD filtrates reproducibly detected 1416, 1468, 1774 D peptides in BALB/c that were reduced or absent in DBA/2, and the presence of 2662, 4559 and 5320 D molecules in DBA/2, the latter two definitely not present in BALB/c. Direct antigen presentation of paternal H-24-restricted paternal peptides (600-1800 D) may prevent. the rejection of the CBAxDBA/2 embryos, and larger sized peptides may bind to immunizing splenocytes and augment abortion mechanisms

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1. Introduction

The DBA/2-mated CBA/J female mouse (the CBAxDBA/2 model) has provided a useful system in which to investigate immune system mechanisms that lead to rejection of the semi-allogeneic embryo, and to define mechanisms that prevent pregnancy failure (Clark et al., 1998, 1999, 2003; Redecha et al., 2009; Clark, 2008). Abortions are currently attributed to suboptimal generation of regulatory T cells (Tregs) that suppress a rejection response

Abdreviations: IVF, in vitro fertilization and embryo transfer: IDO, indoleanime 2.3-dioxygenase; SP, seminal plaima: SELDI-TOW, surfaceenhanced laser densitometry and isolation - time of flight.

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to paternal DBA/2 antigens (Clark et al., 2008a), and the importance of Tregs in preventing rejection in utero has been demonstrated in other mating combinations (Kahn and Baltimore, 2010; Aluvihare et al., 2004; Darrasse-Jeze et al., 2006). Abortions in the CBAxDBA/2 model can be prevented by alloimmunization prior to mating using BALB/c lymphoid cells, which have the same MHC H-2^d as DBA/2, and also by spontaneous immunization during a pregnancy by BALB/c (Chaouat et al., 1988; Clark et al., 1999). Using (BALB/cxDBA/2F1)xDBA/2 backcross-derived lines of mice as a source of cells to preimmunize CBA/J females, Kiger et al. (1985) showed that there were non-MHC genes that prevented abortions and genes that stimulated abortions: the data fitted a model where two independent gene loci, one producing a protective molecule P and the other a product stimulating abortions (5) (Clark, 1999). The P effect was MHC H-2^d restricted, and likely a peptide binding to the groove of the MHC, and S was thought likely to be a peptide although possibly not H-2^d restricted (Clark, 1999). As embryos can express Class I MIIC on trophoblasts, but not Class II MHC, it was proposed that BALB/c has an excess of P>S bound to H-2^d, whereas in DBA/2, the concentration of P was the same as that of S (Hedley et al., 1989). Clark, 1999). By manipulating the ratio of P to S, one should be able to render BALB/c lymphoid cells non-protective and conversely, to render DBA/2 cells protective.

Initially, it was thought that the Class Ib MHC molecule Qa-2 (a homolog of human HLA-G) might be the restricting element for protection, as BALB/cBy that lack Qa-2 apparently did not protect (Kiger et al., 1985). However,





MODEL of DBA/2 SPLENOCYTE ANTIGEN-PRESENTING CELL



Fig. 1. Model illustrating the substitution of Class 1 MHC-presented peptides by peptides in seminal plasma for BAUJ(c and DBA/2 splerocyte antigenpresenting cells in vitro.

further data from (Bobě and Kiger, 1989) suggested that some BALB/cBy lines might immunize against abortions, and for many years in France BALB/c from Charles River have been supplied in the form of BALB/cByJ which are Qa-2°, and immunize effectively against abortions. Thus, H-2d Class ta molecules such as H-2K, H-2D, and/or H-2L had to be the restricting element for presentation of paternal peptide P to prevent abortions. Further, it was possible to perform an exogenous peptide substitution experiment for Class Ia MHC (Bennink and Yewdell, 1989; Su and Miller, 2001: Benke et al., 2006; Brophy et al., 2007), but there was no obvious way of obtaining a significant amount of P and S. Recently, Robertson's group proposed that induction of protective Treg cells in the lymph nodes draining the genital tract begins at the time of mating and is induced by paternal antigens in seminal plasma; thus, the putative P and 5 peptides might be present in seminal plasma (Guerin et al., 2011; Clark, 2011). These Treg cells then localize in the uterus prior to implantation, and counter the activation of potential effector T cells (Guerin et al., 2011; Clark, 2011). However, this model required presentation of paternal antigens to Treg cells by maternal dendritic cells, which would be MHC H-2k restricted. We decided to test BALB/c and DBA/2 seminal plasma for peptides able to bind to and alter the function of H-2st lymphoid cells. The strategy for such experiments is illustrated in Fig. 1 (adapted in part from Gromme and Neefjes, 2002). A "danger" peptide (S) may inhibit the ability of Treg cells to react with the protective peptide (P) by displacing 5, and conversely, protective poptide (P) may displace "danger poptide" (S).

2. Materials and methods

2.1. Mice

CBA/J. BALB/cBy and DBA/2J females or males were obtained at the age of 6 weeks from Charles River. France, and were maintained for 2 weeks in the U782 & U764 joint animal quarters, Clamart, or in the Center Hayem at Höpital Saint Louis, France. To optimize pregnancy success, female CBA/J and male DBA/2 cages were kept adjacent on the same rack for five days before mating, and two days before mating CBA/J females were placed in the cage where DBA/2 males had been previously caged for two days. Matings were then performed as trios (one DBA/2 male, two CBA/] female) for two cages, or for one cage, as a duo (one male, one female). Males were kept in the cage for 3 days. This method ensures in our housing conditions close to a 100% pregnancy rate in each group (five to six mice per group). Mice were checked each morning for a vaginal plug and that day was designated day 0.5 of pregnancy. Mice were sacrificed on day 12.5 of pregnancy. Resorptions were always easily distinguished from viable fetuses.

Splenocytes from BALB/c, DBA/2 and CBA/J mice were obtained as already described using Kontes P22 tissue grinders, washed three times in RPMI 1640 glutamax and then cultured at 10 × 10⁶ cells/ml for 24 h in serum-free RPMI 1640 medium, without the addition of 2-ME or antibiotics, and in the presence eventually of 10 y/ml of brefeldin A (eBiosciences). The control consisted of brefeldin only containing medium also incubated for 24 h, all at 37 °C in a 5% CO₂ humidified incubator in Costar filter corked vials. After 24 h, the cells were centrifuged, and then the pellets were resuspended, and incubated in a 5-ml volume of brefeldin A-containing RPMI medium supplemented with BALB/c or OBA/2 seminal plasma (see below), and maintained thus at 37 °C in the incubator in a Costar minivial for 2 h. Trypan blue dye exclusion test confirmed that the viability bad been maintained above 90% before injection. The cells were then washed three times at 4 °C in 50 ml of brefeldin-containing medium to eliminate any cytokine carry over, before resuspension in 3.0 ml of such medium and injection of 0.5 ml i.p. per mouse.

Seminal plasmas were obtained from eight males per group, centrifuged at 4000 rpm for 10 min in a Heraeus centrifuge beforehand to pellet the cells, and then gently recovered via a Pasteur pipette, placed in a plastic tube and supplemented with RPMI 1640 brefeldin A-containing medium on the cells to reach a final volume of 5 ml. Other controls were medium alone, or splenocytes cultured, pelleted and resuspended and cultured, all in brefeldin A+ medium, under the same conditions as for seminal plasma-treated lymphocytes. 20 mm of seminal plasma-containing medium were further studied after centrifugation at 7000 × g for 30 min through a YM-10 Amicon filter (Millipore Corp, Centricon plus-20 Cat. UCF2L6G02). The 0.5-ml retentate was reconstituted to 20 ml and the 19.5 ml of filtrate was similarly adjusted to 20 ml.

2.2. Proteomic analysis

Proteins and peptides in the <10 kD filtrate of DBA/2 and BALB/c seminal plasma samples were analyzed using surface enhanced laser desorption ionization-time of flight (SELDI-TOF) using the anionic exchange Q10 chip. The protein concentration in seminal plasma filtrate was measured using NanoDrop at 280 nM in triplicate. Next, 5 µl of diluted samples was applied to activated protein chips. The arrays were then analyzed with ProteinChip Reader (series 4000, Bio-Rad). Arrays were exposed to laser energy to detect proteins within the range 500–10,000 D. The bioinformational analysis was performed using Ciphergen Express Client 3.0 software. The principle and usefulness of the methodology is described by Issag et al. (2003).

2.3. Statistics

Data were expressed as already described elsewhere (Clark et al., 2003, 2008b). Analysis for statistical significance of differences between multiple groups of control or preimmunized mouse groups was initially done on individual resorption and implantation rates using a parametric ANOVA as preprogrammed by Ti (Texas Instruments) on a Ti69 calculator with the significance of deviation of individual groups from the control was assessed by a range test (Fisher PLSD) based on five mice per group; a P value ≤0.05 was considered significant. Although there is very little difference between parametric and nonparametric statistical values for resorption rates, because implantation number variation can unfairly weight resorption rates from some mice, the standard method of pooling the number of resorptions and total number of implantation sites



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Fig. 2. (A) Resorption rates (RR) in DBA/2-mated CBA/) mice are shown after various pre-mating treatments. CTRL represents pooled data from mice treated with RPMI or RPM - brefeldin A, totaling 89 implantations from 13 mice (RR - 24/85). B. Preimmunization with brefeldin A-treated BALB/c splenocytes (RR = 3/34): 8+8. brefeldin-treated BALB/c splenocytes incubated in BALB/c seminal plasma (RR = 1/32): B+D, brefeldin A-treated EALB/c splenocytes incubated with DBA/2 seminal plasma (RR - 7/21); D trefeldin-treated DBA/2 splenocytes (RR - 5/24); D+B, brefeldin-treated DBA/2 splenocytes treated with BALB/c seminal plasma (RR = 3/39); C+B, brefeldin A-treated CBA/J splenocytes incubates with IIALII/c seminal plasma (RE - 17/41). The protein concentration in unfractionated IIAEB/c SP was 0.55 giml and in DBA/2 SP 0.56 g/ml. (B) The effect of DBA/2 splenocytes treated with the «10kB (RR = 2/24) and >10kD (RR = 10/33) fractionation of BALIJ/c seminal plasma, Medium control (RR - 10/34), (C) The result with BALR/c cells treated with the <10kD (RR-21/63) and >10kD (RR-15/75) fractions of DBA/2 seminal plasma: medium control (RR - 9/27). Ilrefeldin A-treated CBA/J splenocytes incubated with the <10kD fraction is also shown (RR - \$/28). The 95% confidence interval is shown for the control group determined from the binomial. "Significant reduction in the resorption (abortion) rate (P<0.05) as described in the Section 2. **Significant increase in the abortion rate.

from individual mice to calculate resorption rates was used, and the significance of differences seen in ANOVA was verified by use of non-parametric χ^2 or Fisher's Exact test where appropriate (Clark et al., 2008b). Only differences confirmed as significant by nonparametric analysis were deemed to be significant. Where only two groups were compared, the nonparametric χ^2 or Fisher's Exact test were used. Since there was no difference between control groups treated with RPMI and RPMI+brefeldin (see Section 3). these groups were pooled to improve the precision of the estimate of baseline resorption and implantation rates.

3. Results

Fig. 2A shows the number of resorptions in DBA/2mated CBA/J mice following different treatments determined on day 13.5 of gestation. In the control-treated mice,



five received RPMI and had a mean resorption rate ± SEM of 28.0 ± 5.6 which was not different from eight mice receiving RPMI + brefeldin, 26.5 ± 2.7 based on individual mice; the pooled group result for RPMI was 9/32-28.1% and for RPMI + brefeldin 26.3%, and these differences were not significant based on χ^2 . Therefore, the results were pooled giving 24/89 (27.0%, binomial 95% confidence interval 17.8-36.2). This provides a guide to assessing significant differences, but to be sure one must calculate the P value from the raw data provided in the figure legend. The resorption rate was significantly reduced when the females had been pre-immunized with BALB/c cells, or BALB/c cells incubated with brefeldin (B) and BALB/c seminal plasma in the incubation mixture (B+B). In contrast, where DBA/2 seminal plasma had been substituted, the BALB/c cells completely lost their ability to protect against abortions. Brefeldin-incubated DBA/2 cells were also nonprotective (D), but acquired significant protective activity when BALB/c seminal plasma had been added to the incubation medium (D+B). Importantly, pre-incubation of CBA/J splenocytes with brefeldin and BALB/c seminal plasma (C+B) failed to reduce the abortion rate, although the higher resorption rate in this group fell outside the upper 95% confidence limit of the control the increase in resorption rate was not significant based on nonparametric testing (χ^2 without Yates' correction = 2.732, P> 0.05 two-tailed null hypothesis that the resorption rate was not different in the C+B-treated mice compared with the control group; the variances were also not different using the F statistic to compare the two groups). We concluded that there were substances in seminal plasma that could determine the ability of H-2^d splenocytes to immunize against abortions, and the protective effect was H-2^d and not H-2^k restricted.

5. 4 3 2 0 CTRI в B+B B+D D D+B C+B в С 8 6 BSNOW NAR NOUS STOR NOR STOR CTRL -158 1100 **CTR** 124 HIN 085(10) GADUPS GROUPS Fig. 3. (A) Implantations per mouse are shown corresponding to the pan-



To test whether the protective substance was of low molecular weight, BALB/c seminal plasma was fractionated into a <10 kD and >10 kD pool as described in the Section 2. As shown in Fig. 2B, treatment of DBA/2 splenocytes with the <10 kD fraction conferred the ability to protect against abortions. Conversely, separation of DBA/2 seminal plasma and treatment of BALB/c cells with the <10-kD fraction significantly boosted the abortion rate (Fig. 2C). This effect was not statistically significant when CBA/J splenocytes were treated.

Resorptions typically begin on day 9.5 in the CBAxDBA/2 model, and resorptions persist until scoring on day 13.5 of pregnancy. Earlier losses may have a different mechanism of causation and result in a reduced number to implantations (live or resorbing) on day 13.5 of pregnancy. Fig. 3A shows the effect of treatment on the number of implantations per mouse. It can be seen that immunization with BALB/c cells pre-treated with DBA/2 seminal plasma or treatment with brefeldin-treated DBA/2 cells reduced the number of implantations. In Fig. 3B, it can be seen that neither the <10-kD nor >10-kD fractions of BALB/c seminal plasma altered the number of implantations significantly, but in Fig. 3C, the BALB/c cells treated with the <10 kD fraction of DBA/2 seminal plasma showed a small reduction in implant number, as did CBA/J cells treated with the <10-kD fraction, but this did not reach statistical significance.

As shown in Fig. 4, SELDI-TOF-MS analysis showed several peaks in the size range of 9 amino acid peptides 9 (800-1800 D) that were either present only on BALB/c SP (1458, 1669, and 1744D) or present at a higher concentration (based on the magnitude of the µA signal) than in DBA/2 SP(1417 D). In contrast, DBA/2 contained peptides at 4559 and 5320 D not identified in BALB/c; there was a 2662 peak less convincing than those at 4559 and 5320 in DBA/2 and a similar blip in BALB/c at 2662 not declared significant by the software program. (Both DBA/2 and BALB/c exhibited peptides at 9125/9059 D, the µA signal was greater for DBA/2, and there was also 10339 proteins at a similar concentration and a 10567 D peak in DBA/2.) There were also a number of small molecules 600 D± in both, with an apparently higher concentration in BALB/c. We concluded that the data were compatible with the presence of a higher concentration of peptides in BALB/c SP that could bind to MHC Class I H-2^d and explain the protective effect seen.

4. Discussion

The data in this paper confirm the ability of BALB/c seminal plasma components to prevent abortions in the CBAxDBA/2 model by pre-treating brefeldin A pretreated H-2^{d+} splenocytes and washing, so that minimal seminal plasma was transferred. As the protective component was <10 kD by Amicon filtration, the protective moiety is consistent with the effect being induced by binding of paternal non-MHC peptide(s) to the binding groove of MHC. It was possible to render otherwise non-protective DBA/2 splenocytes protective by loading with BALB/c seminal plasma, and conversely, to render otherwise protective BALB/c splenocytes ineffective by loading with DBA/2 seminal plasma components. There was no effect when CBA/J splenocytes were used, and the H-2^d restriction makes it unlikely that either cytokines present in seminal plasma or prostaglandins could be responsible. SELDI-TOF-MS analysis was consistent with there being peptides at the 600-1800D range in BALB/c seminal plasma at a greater concentration than in DBA/2, and one of these could be responsible for the protective effect. From the immunogenetic data it had been deduced that DBA/2 likely has genotype p-/s-, and BALB/c genotype for the protective gene is 'pp', so the peptide at 1416 present at twice the concentration in BALB/c compared with DBA/2 (owing to a gene dose effect) could represent the protective moiety. On this basis, one would predict that the ratio of peaks in BALB/c:DBA/2 for the protective "P" effect should be approximately 2. The 791 peptide had a ratio of 2.08, the 1145 peptide had a ratio of 2.18, the 1415 peptide had a ratio of 2.1, and the 1666 peptide had a ratio of 2.14. All of these would be candidates for the proposed "p" gene product P

We found that DBA/2 seminal plasma, in contrast to BALB/c seminal plasma, contained a <10-kD activity that conferred upon otherwise protective BALB/c cells the ability to boost the abortion rate, and SELDI-TOF-MS analysis indicated that the molecules responsible were likely larger than typical 9 amino-acid P peptides (estimated MW range 600-1800 D) of the type that bind to the groove in Class I MHC and which correlated with protection against abortion (illustrated in Fig. 1). It had been assumed that the genotype of BALB/c is pp/-, but reanalysis of the immunogenetic data indicates that a genotype of pp/s- provides a better fit to the frequency of protective, nonprotective, and abortion-augmenting recombinant inbred lines (Kiger et al., 1985; Clark, 1999). On this basis, one would predict that the ratio of peaks in BALB/c:DBA/2 for the abortionstimulating effects attributed to 5, close to 1. In this regard, the molecule(s) at 10339 D occurred at a ratio of 0.8, which is close to the value of 1 predicted above for S. Further, in the CBAxDBA/2 model, NK yoT cells that produce TNF- and IFN-y have been proposed to promote abortions, and the yð TcR can recognize larger peptides in the 2600-10,000 range in a non-MHC-restricted manner (Arck et al., 1997; Clark and Croitoru, 2001; Born et al., 2011). If P were bound to H-2^d in the >10 kD component of seminal plasma, less would pass the filter so that with BALB/c seminal plasma, P could be >S, but at a ratio of less than 2:1, and with DBA/2, P would be <5, which would explain the detection of augmentation of abortions by fractionated, but not unfractionated DBA/2 seminal plasma. The reason for the fact that unmodified DBA/2 splenocytes and DBA/2 seminal plasma-treated BALB/c splenocytes did not boost abortion rates could be due to the abortogenic factor(s) being bound to carriers in unfractionated seminal plasma. The presence of abortogenic activity in DBA/2 seminal plasma was only demonstrable after Amicon filtration, possibly explained by the abortogenic factors being freed from antagonistic binders >10 kD. Further, when CBA/J splenocytes were treated with BALB/c seminal plasma, absent H-2^d only 5 (which is not MHC-restricted) would bind. In that situation, one would expect a boost in abortion rates, and assuming a unidirectional one-tailed null hypothesis, the P value compared with the control, which was not significant using a two-tailed test did achieve P<0.05 by a one-tailed χ^2 test.



Fig. 4. SELDI-TOF mass spectrometry of <10-kD DBA/2 and BALB/c seminal plasma filtrate. The results are ordered with respect to molecular weight on the X-axis and DBA/2 is shown in the top part of the panel (spot G) and BALB/c (spot H) in the bottom part of each panel. Only panels where signals were identified are shown. The magnitude of the signal is shown in the Y-axis in µA. The scale of the panels was adjusted in illustrate significant peaks that were identified and labeled by the software.

One caveat is BALR/c cells treated with <10 kD DRA/2 seminal plasma had an increased rate of abortions, but treated CBA/J did not show an effect in Fig. 2C that achieved statistical significance. However, this could be explained if S had more affinity for H-2⁴⁺ cells than H-2^{k+} cells and was at a low enough concentration to detect this: a 10-kD filter could be expected to impair passage of molecules of 10339 D.

Mass spectrometry does not identify molecules and isolation using reverse-phase HPLC of the <10 kD seminal plasma filtrates, correlation with the SELDI-TOF peaks, and sequencing followed by direct testing of each individual species for bioactivity in vivo are necessary to establish what molecules are altering the outcome of the pregnancy, to confirm the size of the abortogenic molecule, to show that the molecule activates NKybT cells, and to determine to what extent Class I MHC type may affect binding to lymphoid cells. The observation of two moieties, one boosting the abortion rate and one reducing it are consistent with the original conclusions that arose from immunogenetic studies (Kiger et al., 1985; Clark, 1999). Concerning the protective effect in BALB/c seminal plasma, one does not know if seminal plasma alone in vivo can alter the immune system of the future mother, or if an embryo must be gencrated.

A second observation shown in Fig. 3 was that the number of implantations noted at day 13.5 of pregnancy could he reduced by DBA/2 cells and by cells treated with DBA/2 seminal plasma. It was not possible to determine with the number of mice available if the small reduction in implantation number was due to a <10 kD molecule in seminal plasma, or to determine if the effect was H-2° restricted or not. Notably, the >10 kD fraction of DBA/2 seminal plasma had no effect on the number of implantations per mouse. These observations taken together suggest that the protective effect of the putative peptide in the <10-kD fraction of BALB/c seminal plasma might be more potent. Different immune mechanisms are thought to be involved in early losses. Further studies will be required to identify the active moiety and to investigate the mechanism of these effects.

Paternal minor histocompatibility antigens appear to play a role in early post-implantation loss in mice fed 1-MT to block IDO (Mellor et al., 2001), and Hill's laboratory has reported that T cells from women respond in vitro to antigens in sperm (which are coated with seminal plasma) or in trophoblast preparations in a manner that correlated with subsequent success or failure after IVF (Ginsburg et al., 2005). This would be homologous with a response in the CBA/J mouse to abortogenic antigens presented by H-2k antigen presenting cells. In the model proposed by Sarah Robertson's lab, paternal antigens in seminal plasma induce antigen specific Treg cells in the uterine draining lymph nodes, and these Tregs home to the uterine lining where they can react with similar embryo-release paternal antigens presented by maternal dendritic cells (Guerin, et al., 2011). However, we were unable to protect against abortions using CBA/J splenocytes loaded with protective BALB/c seminal plasma peptides. This observation is noncongruent with the Robertson model, where recognition of paternal peptides would be H-2^k restricted (Guerin et al., 2011). However, one cannot exclude dose effects, or alternative antigenic stimulation signals: as there are small numbers of H-2d* lymphomyeloid cells in ejaculate, and it is possible that maternal Tregs are induced or recruited by these cells presenting paternal peptides, or that the peptide presentation effect is enhanced by the coexistence of an allogeneic reaction, just a bystander carrier-like effect can be observed for soluble antigen-induced reactions in the presence of an allogeneic reaction. There was enough free peptide P not bound to H-2^d in BALB/c SP to pass through the Amicon filter, although the levels seen in Fig. 4 could underestimate the total amount present in unfractionated seminal plasma. Recently 5hima et al. (2012) reported that seminal plasma was required to prevent early loss plus resorptions of embryos in DBA/2-mated BALB/c mice, where the MIs1a of DBA/2 was the inciting paternal antigen-causing loss. Although this model is compatible with maternal APC presentation of paternal antigens in seminal plasma, as the mice are syngeneic at MHC, it is not possible to exclude MHC-restricted presentation by paternal MHC in seminal plasma.

An important caveat is that seminal plasma is not required for successful pregnancy. Transfer of embryos fertilized in vivo or in vitro is known to be quite successful in mice and humans, and in humans, exposure to seminal plasma has only a small effect by improving the implantation rate (Coulam and Stern, 1995; Tremellen et al., 2000). It has been suggested that paternal antigens expressed by the "fetal allograft" (i.e. semiallogeneic embryo) are recognized by maternal T cells exclusively through the indirect pathway of antigen presentation, and that this pertained to a suboptimal maternal T cell reaction to antigens expressed by the placenta alter day 7.5 of pregnancy (Erlebacher et al., 2007). The suboptimal response may be due to a population of Treg cells activated by indirect presentation of paternal placental antigens at mid-pregnancy (Samstein et al., 2012). Our data suggest that there might be direct antigen recognition in the preimplantation stage of pregnancy when seminal plasma may recruit and activate Treg cells (Guerin et al., 2011). Whether the latter are distinct from Tregs activated at mid-pregnancy remains to be determined. In the absence of seminal plasma, Treg cells would have to react to paternal peptide + paternal Class 1 MHC H-2^d complexes derived from pre-implantation embryos, as paternal Class I MHC, but not Class I MHC, antigens are expressed by these embryos. Secreted or shed antigens could pass though the zona pellucida in soluble or exosomal form and alter the maternal immune system prior to implantation. There is consensus (with the exception of one report of limited expression by electron microscopy) that the hatched blastocyst at implantation has shut off expression of class I MHC and there is no surface expression of classical MHC at the time of implantation (Hedley et al., 1989), Further, there is no mRNA for paternal MHC in the mouse until day 7.5 of pregnancy (Hedley et al., 1989), so a soluble complex arising from pre-implantation embryos must be postulated, and Tregs must be able to be generated without the participation of maternal DCs. In the MIs model mentioned above, MLs may be a retrovirus that may be expressed by early trophoblasts, and it unclear whether it is restricted by maternal or paternal MHC, so one cannot exclude the possible relevance of indirect antigen presentation (i.e., cross-presentation). Such antigens are 'superantigens' that act by binding to Class II MHC and TcR independent of the "P" amino acid peptide-binding groove of MHC. However, they are larger than the molecules we found in our <10-kD filtrates, and thus are unlikely to explain our observations. With respect to identifying the <10-kD molecules that alter pregnancy outcome in the CBAxDBA/2 model, it could be difficult to accumulate sufficient material for biochemical characterization from embryos, but as the putative molecules that reduce or boost the abortion rate are present in seminal plasma; for which there is an abundant supply, it should be possible to characterize the latter, and then to look at preimplantation embryos for the former. This could greatly enhance our understanding of the molecular triggering events leading to the success or failure of pregnancy.

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7.4. Summary in French

Rôle du Granulocyte-Colony Stimulating Factor (G-CSF) dans le Processus Implantatoire, chez la Femme et en Modèle Murin

1. Introduction

L'immunologie de la reproduction est une discipline qui réunit les principes généraux de l'immunologie générale et les aspects spécifiques de la reproduction et du développement. Les Colony Stimulating Factors (CSFs) sont une illustration des possibilités d'application médicale de ce domaine. Dans la famille des CSFs, le Granulocyte-Colony Stimulating Factor (G-CSF) apparait comme une cytokine majeure pouvant intervenir dans la thérapie de pathologies en reproduction, en particuliers celles liées à un défaut d'implantation embryonnaire. Dans ce travail, à travers une revue des CSFs dans la reproduction, une étude des gènes cibles du G-CSF dans l'endomètre Humain ainsi qu'une étude des effets de la supplémentation systémique en G-CSF dans un modèle murin pro-abortif, nous avons essayé d'approcher certains mécanismes d'action de cette cytokine dans le processus implantatoire via le biosenseur endométrial.

1.1. Rappels sur l'Implantation Embryonnaire

Malgré plus de trente ans de progrès majeurs en Assistance Médicale à la Procréation, l'implantation embryonnaire humaine reste un facteur limitant et un phénomène impossible à étudier in situ, étant donné des considérations éthiques évidentes. Actuellement, nous savons que, chez la femme, après une fécondation naturelle et un trajet à travers une des trompes utérines, l'embryon entre dans la cavité utérine au cinquième jour, au stade de blastocyste. L'implantation se déroule en plusieurs étapes successives : apposition, adhésion et invasion. Le déroulement physiologique de ces différentes étapes nécessite une préparation endométriale spécifique. Les cellules d'origine trophoblastiques sont effectivement particulièrement invasives, souvent comparé dans leur potentiel invasif à une invasion néoplasique, pouvant survenir sur tout type de tissu, sauf l'endomètre qui contrôlera cette invasion. Durant le cycle, l'endomètre ne permet l'implantation embryonnaire que pendant une période précise de cinq jours appelée « fenêtre d'implantation », du cinquième au neuvième jour après l'ovulation, ce qui definit le concept de réceptivité utérine

La réceptivité endométriale pendant cette fenêtre implantatoire est liée aux changements structuraux et fonctionnels qui surviennent de manière cyclique, sous l'effet des hormones ovariennes. Les modifications structurales concernent l'épithélium de surface (apparition de pinopodes et de microvillosités), les glandes (deviennent contournées et sécrétantes), les artères appelées spiralées, et le stroma (avec une matrice lâche faites de cellules decidualisées). A l'échelle moléculaire, on observe la disparition de systèmes antiadhésifs (glycocalyx, mucines) et l'apparition de molécules d'encrage et de migration cellulaires (intégrines). Ces changements concernent enfin la population de cellules immunitaires locales avec un switch immunitaire fondamental. D'une immunité acquise protégeant pendant la majorité du cycle l'endomètre contre les attaques microbiennes, une immunité innée spécifique se développe permettant l'implantation d'un embryon semi-allogénique.

L'endomètre decidualisé est alors décrit comme un biosenseur capable d'établir un dialogue avec le blastocyste. Des échanges de signaux embryonnaires et maternels vont ainsi permettre une implantation harmonieuse. D'une part l'endomètre doit être capable d'interagir avec l'embryon, d'autre part, l'embryon doit être capable de moduler cette réaction maternelle afin d'établir un environnement endométrial favorable à l'implantation. Du point de vue immunologique et en simplifiant à l'extrême, une implantation embryonnaire réussie nécessite une réaction pseudo inflammatoire initiale (permettant les premières étapes d'encrage de l'embryon) transitoire suivie d'une modulation de l'immunité locale vers un état de tolérance local (évitant le rejet de l'embryon). Cet équilibre complexe et délicat est maintenu localement grâce à de nombreux acteurs : cytokines, facteurs de croissance, molécules HLA spécifiques, cellules immunitaires locales telles les utérine Natural Killers (uNK), dendritiques et T régulatrices. Ces interactions répétées entre mère et conceptus permettent non seulement la réussite des premières étapes de l'implantation, mais aussi le contrôle de l'invasion embryonnaire et la placentation ultérieure.

Apres l'adhésion à l'endomètre maternel, les cellules trophoblastiques du blastocyste se différencient en cytotrophoblaste et syncytiotrophoblaste. Grace à la sécrétion d'enzymes lytiques et de facteurs pro-apoptotiques, l'embryon progresse dans le stroma endométrial. La formation de villosités choriales primaires, secondaires et tertiaires a lieu au cours de la troisième semaine de gestation. Parmi les villosités tertiaires, certaines atteignent la lame basale endométriale et constituent les villosités d'ancrage, à partir desquelles une population cellulaire extrêmement invasive appelée cytotrophoblaste extravilleux va débuter sa migration. Certaines de ces cellules poursuivent une migration interstitielle jusqu'au tiers proximal du myomètre, et d'autres ont un trajet endovasculaire, envahissant et remodelant la paroi des artères spiralées préalablement decidualisées, permettant l'établissement d'un système vasculaire à faible résistance et optimisant ainsi les échanges materno-fœtaux. Ces processus aboutissent à la mise en place d'un placenta hémochorial.

Du point de vue endométrial, un défaut de décidualisation ou de réceptivité, une hyper activation immunitaire locale, ou un défaut de contrôle de l'invasion embryonnaire peuvent induire une implantation défectueuse avec des conséquences diverses. Des dérégulations profondes et précoces peuvent induire des tableaux cliniques tels que des fausses couches à répétition ou des échecs d'implantation embryonnaire. Une invasion embryonnaire limitée peut avoir pour conséquence obstétricale un retard de croissance intra utérin ou une prééclampsie. Au contraire, un défaut de contrôle de l'invasion embryonnaire peut être à l'origine de pathologie obstétricale hémorragique due à un défaut de délivrance placentaire, telle que le placenta accreta.

1.2. Rappels sur l'Immunologie de la Reproduction

L'immunologie en général, traite de la reconnaissance du soi et du non soi, permettant la défense et l'intégrité de l'organisme. On y distingue classiquement l'immunité innée de l'immunité adaptative. L'immunité innée, via des cellules telles que les macrophages, les

cellules dendritiques, les neutrophiles, les Natural Killers et les lymphocytes autres que B et T, est responsable d'une réponse immunitaire immédiate et antigène indépendante. L'immunité adaptative assure une réponse antigène spécifique, via les cellules présentatrices d'antigènes, leur reconnaissance par les lymphocytes T avec l'intervention du système HLA, la production d'anticorps spécifiques par les lymphocytes B et la mise en place de cellules mémoire.

Ces systèmes immunitaires complexes et plastiques interagissent et sont régulés par des molécules solubles secrétées appelées chemokines et cytokines. L'environnement cytokinique des cellules immunitaires est décisif dans leur différenciation et leur orientation, vers une réponse pro-inflammatoire ou tolérogène.

Du point de vue de l'immunologie générale, l'implantation embryonnaire et la grossesse sont des évènements exceptionnels qui ne peuvent s'expliquer par les mécanismes classiques de la transplantation. L'immunologie de la reproduction est un domaine qui inclut les différents aspects de ces deux spécialités.

Les premières hypothèses tentant d'expliquer « le paradoxe de l'allogreffe fœtale » datent des années 1950, décrivant le fœtus comme immunologiquement neutre ou la mère comme immunodéprimée lors de la grossesse. Les hypothèses suivantes dans les années 1970 cherchaient des mécanismes généraux d'immunotolérances.

Mais dès les années 1980, l'attention s'est particulièrement portée sur des mécanismes locaux. Le premier mécanisme exploré à l'interface mère-conceptus a été l'immunosuppression locale avec la présence de cytokines tolérogènes. L'immunogenicité particulière des annexes de l'embryon avec la présence de molécules HLA monomorphes (HLA-G) ou a polymorphisme réduit (HLA-C) a aussi été rapporté comme participant à cette immunomodulation locale.

L'existence de cellules immunitaires clés appelées les uterine Natural Killers (uNK) et leur interaction avec le HLA-C ont parallèlement été décrit comme indispensable au remodelage vasculaire des artères spiralées, permettant une placentation satisfaisante ultérieure.

Par ailleurs, la présence de certaines cytokines dans cette interface a été démontrée comme nécessaire non seulement à l'établissement de l'équilibre immunitaire locale mais aussi

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pour la croissance embryonnaire, fœtale et placentaire ; ceci constitue la « théorie de l'immunotrophisme ».

Parmi les nombreuses cytokines présentes à cette interface, l'existence de facteurs inflammatoires a été démontrée comme nécessaire à l'implantation embryonnaire. L'implantation n'est plus décrit comme nécessitant uniquement un environnement tolérogène, mais une suite d'évènements immunitaires régulés dans une chronologie spécifique. La famille des CSFs, et en particulier le G-CSF, font partie de ces réseaux cytokiniques clés à l'interface mère-conceptus.

1.3. Objectifs et Hypothèse

Dans ce travail, nous avons essayé de contribuer à la compréhension du rôle des CSFs en reproduction et en particulier l'action du G-CSF dans l'implantation embryonnaire, notre hypothèse étant que le G-CSF pourrait intervenir dans les phénomènes implantatoires en modulant le biosenseur endométrial avant l'arrivée du blastocyste.

Pour cela, nous présentons un article de revue sur les CSF en reproduction, un article de recherche portant sur l'effet de la supplémentation en G-CSF sur des gènes cibles dans l'endomètre humain et un article de recherche sur les effets de la supplémentation systémique en G-CSF sur l'implantation embryonnaire dans des modèles murins pro-abortif et fertile.

2. Les Colony Stimulating Factors (CSF) dans la Reproduction

2.1. Les Colony Stimulating Factors (CSFs)

La famille des CSFs (Colony Stimulating Factor) comprend: le CSF-1 ou M-CSF (Macrophage-Colony Stimulating Factor), le CSF-2 ou GM-CSF (Granulocyte-Macrophage-Colony Stimulating Factor), et le CSF-3 ou G-CSF (Granulocyte-Colony Stimulating Factor). Ces cytokines ont été étudiées à partir du milieu des années 1960 et nommés d'après leur action sur la prolifération et la différenciation des leucocytes. Leur implication dans la reproduction a été portée depuis le début des années 1970, quand une activité de type CSF a été identifiée dans le placenta humain et murin.

2.2. Localisation des CSFs dans l'Appareil Reproducteur

Tous les CSFs et leurs récepteurs correspondants ont été localisés le long de l'appareil reproducteur féminin, mais chacun a des sites d'expression spécifiques. À l'interface fœtomaternelle, le GM-CSF et son récepteur ont été les plus étudiés. Dans l'ovaire, la granulosa et le liquide folliculaire, le G-CSF semble soulever plus d'intérêt dans la recherche fondamentale et les applications médicales. Dans le plasma séminal, le GM-CSF est à nouveau le plus étudié parmi les CSFs, notamment en raison de son action sur l'endomètre avant l'implantation.

2.3. Les fonctions des CSFs dans la Reproduction

Le M-CSF et surtout le G-CSF sont décrits comme impliqués dans les mécanismes de l'ovulation. Dans implantation embryonnaire, la croissance placentaire et le développement embryonnaire, le GM-CSF a la plus grande implication, à travers son rôle d'immunomodulateur, ses effets angiogéniques et trophiques locaux.

2.4. Les Applications Médicales des CSFs
Les CSF ont été proposés comme biomarqueurs. Les variations sériques du G-CSF et du M-CSF lors de l'ovulation et de la stimulation ovarienne suggèrent une utilisation possible de ces taux sériques comme marqueurs prédictifs des résultats en FIV. Des taux sériques élevés de CSF ont également été décrites dans diverses pathologies de la reproduction telles que la pré-éclampsie, l'accouchement prématuré ou les fausses couches spontanées à répétition. Mais surtout, le taux de G-CSF folliculaire a été présenté comme un biomarqueur préconceptuel du potentiel implantatoire de l'ovocyte correspondant et est actuellement en développement pour l'application clinique.

Compte tenu de ses effets trophiques, la supplémentation en GM-CSF dans les milieux de culture embryonnaires en cas d'assistance médicale à la procréation est en cours d'évaluation. Des études récentes suggèrent un taux de grossesses évolutives supérieur avec cette supplémentation, en particulier chez les patientes ayant des antécédents de fausses couches spontanées à répétition.

Enfin, la supplémentation en G-CSF semble être actuellement l'une des thérapies les plus innovantes en médecine de la reproduction. Quelques tentatives de supplémentation systémique en G-CSF ont déjà été rapportées en cas de dysovulation. L'instillation intrautérine de G-CSF a été suggérée en cas de troubles trophiques de l'endomètre résistant aux traitements habituels. Mais surtout, la supplémentation systémique en G-CSF est actuellement évaluée pour améliorer la réceptivité utérine, chez les patients ayant des antécédents de fausses couches spontanées à répétition ou d'échec d'implantation embryonnaire.

2.5. Article Dédié aux CSFs dans la Reproduction

Un article de revue original dédié à ce sujet et intitulé « Colony Stimulating Factors 1, 2, 3 and Early Pregnancy Steps: From Bench to Bedside » a été soumis au « Journal of Reproductive Immunology », et est présenté dans les annexes de ce travail.

3. Les Effets du Granulocyte-Colony Stimulating Factor (G-CSF) sur l'Endomètre Humain

3.1. Le Granulocyte-Colony Stimulating Factor (G-CSF) en Reproduction

La supplémentation en G-CSF apparaît aujourd'hui comme l'une des thérapies potentiellement innovante en médecine de la reproduction. Cette thérapie est proposée avec différents modes de supplémentation : soit systémique, par administration souscutanée, ou locale, par instillation intra-utérine. Les indications médicales ne sont pas strictement définies. Le G-CSF est actuellement utilisé dans différents cas de troubles de la reproduction tels que fausses couches spontanées à répétition inexpliquées, les échecs d'implantation embryonnaire répétés ou les troubles trophiques de l'endomètre.

Le G-CSF et son récepteur ont été localisés dans le tractus génital féminin. Les effets proinflammatoires, immuno-modulateurs ou trophiques du G-CSF ont été évoqués dans différents modèles, mais les mécanismes d'action par lesquelles le G-CSF devrait influer positivement sur l'implantation embryonnaire sont largement inconnus. L'objectif de la présente étude était d'identifier les possibles voies moléculaires endométriales influencées par le G-CSF, avec les gènes cibles impliqués sélectionnés à partir d'une étude préalable en microarray et l'utilisation d'un modèle ex vivo de microhistoculture d'endomètre humain précédemment décrit.

3.2. Utilisation d'une Précédente Etude de Microarray Endométrial

Des voies et des interactions moléculaires hypothétiques influencées par le G-CSF dans l'endomètre ont été sélectionnées grâce à une étude précédente en microarray. Cette étude avait été réalisée en comparant l'expression de gènes dans l'endomètre lors de la fenêtre d'implantation, entre des femmes fertiles, les patientes ayant des antécédents d'échecs répétés d'implantation embryonnaire et les patientes avec une histoire de fausses couches spontanées à répétition idiopathiques. Cette analyse avait suggéré de nombreuses dérégulations d'expression pré-conceptuelles dans l'endomètre. Lors de l'analyse de ces voies complexes impliquant le G-CSF, l'expression de certains gènes semblait

spécifiquement dérégulée. L'article précédent consacré à cette étude en microarray est disponible dans les annexes.

Parmi les gènes dont l'expression endométriale était fortement dérégulée et qui hypothétiquement dépendait de l'action du G-CSF, nous avons identifié des gènes impliqués dans la régulation immunitaire, dans le système de coagulation et des intégrines. Nous avons sélectionné les gènes suivants, comme des cibles potentiels du G-CSF dans l'endomètre : G-CSF Receptor (G-CSFR), l'intégrine alpha-V / bêta-3 (ITGB3) impliquée dans la migration cellulaire et l'implantation embryonnaire, le Plasminogen Activator Urokinase Receptor (PLAUR) décrit dans l'interaction avec les intégrines et impliquée dans la migration cellulaire, la Thymidine Phosphorylase (TYMP) impliquée dans l'angiogenèse locale, CD40 et CD40 ligand (CD40L) impliqués dans le contrôle de la prolifération cellulaire.

Nous avons d'abord confirmé les variations de l'expression endométriale de ces gènes cibles chez les femmes fertiles et les patients atteints de troubles de la reproduction. Puis, nous avons utilisé un modèle ex vivo pour illustrer les effets de la supplémentation en G-CSF sur l'expression endométriale de ces gènes.

3.3. Utilisation d'un Précèdent Modèle Ex Vivo de Microhistoculture Endométriale

La microhistoculture endométriale utilisée dans notre étude a déjà été décrite comme un modèle ex vivo fonctionnel. Les échantillons d'endomètre ont été prélevés lors de la fenêtre implantatoire. Ils ont été placés sur des gels d'éponge de collagène, dans un milieu spécifique, quotidiennement supplémenté en estradiol et progestérone. Dans ce modèle, la fonctionnalité et de la différenciation cellulaire sont conservées pendant cinq jours. L'article précédent consacré à ce modèle est présenté dans les annexes.

Sur ces cultures ex vivo, nous avons utilisé une supplémentation pendant trois journées consécutives. Pour la stimulation avec du G-CSF, les cinq conditions de culture étaient les suivantes : du G-CSF à 20 ou 100 ou 200 ng / ml, ou de l'anticorps bloqueur anti G-CSF à 3 pg/ml, et une condition de culture témoin sans G-CSF ni anticorps. Pour cibler spécifiquement l'action endométriale du G-CSF, les mêmes expériences ont été réalisées

avec du Granulocyte-Macrophage-CSF (GM-CSF) à 20 ou 100 ou 200 ng/ml ou de l'anticorps bloqueur anti GM-CSF à 3 pg/ml.

Les protéines recombinantes ou les anticorps ont été ajoutés au milieu de culture chaque jour, pendant trois jours consécutifs. Après leur incubation de trois jours, les échantillons d'endomètre ont été prélevés et placés dans une solution de stabilisation de l'ARN, pour une extraction ultérieure avec RT-PCR quantitative, afin d'évaluation des variations d'expression des gènes cibles considérés.

3.4. Article Original Dédié aux Effets du G-CSF dans l'Endomètre Humain

Un article de recherche original dédié à ce sujet et intitulé « Granulocyte-Colony Stimulating Factor Related Pathways Tested on an Endometrial Ex-Vivo Model » a été soumis et accepté au journal « PlosOne », et est présenté dans les annexes de ce travail.

4. Les Effets du Granulocyte-Colony Stimulating Factor (G-CSF) sur un Modèle Murin Pro-Abortif

4.1. Le Modèle Murin Pro-Abortif CBA/J x DBA/2

Il existe plusieurs modèles murins pour l'étude de l'implantation embryonnaire et ses pathologies. Parmi ces modèles, l'accouplement de femelles CBA / J avec des males DBA / 2 montre une forte incidence de résorption embryonnaire en comparaison avec les femelles CBA / J accouplées aux males Balb / c, considérés comme des croisements contrôles. La résorption de l'embryon chez la souris est considérée comme équivalente à la fausse couche précoce spontanée chez la femme. Ce croisement murin pro-abortif a par ailleurs récemment été décrit comme offrant un modèle murin de la pré-éclampsie.

Les mécanismes de résorption embryonnaire dans ce modèle sont décrits comme indépendants du CMH et semblent être liés à certains peptides présents dans le plasma séminal paternel, interférant avec le biosenseur endométrial pré-implantatoire. Une publication récente sur ce sujet précis est proposée dans les annexes.

En médecine de la reproduction, la supplémentation en G-CSF est proposée comme un traitement efficace pour réduire les taux de fausses couches spontanées ou augmenter le taux d'implantation embryonnaire, tandis que ses actions sur l'implantation restent effectivement peu documentées. Notre objectif était d'identifier les effets de l'administration du G-CSF, par la voie systémique, sur les taux d'implantation et de résorption embryonnaire. Nous avons donc testé les effets de la supplémentation systémique du G-CSF à des doses différentes dans les deux croisements.

4.2. Les Schémas de Supplémentation en G-CSF

Au cours de la période post-ovulatoire et pré-implantatoire, entre le jour 1 et le jour 4 postcoïtum, les injections intra-péritonéales de G-CSF ont été réalisées chez des femelles CBA / J, soit après le croisement pro-abortif (CBA / J x DBA / 2) ou le croisement fertile (CBA / J x Balb / c). Les femelles ont été examinées chaque matin pour vérifier la présence d'un bouchon vaginal et designer le 1^{er} jour de la gestation. Chaque femelle a ensuite été inclue dans un groupe spécifique tel que décrit ci-dessous.

Deux protocoles de supplémentation de G-CSF ont été utilisés : dans le premier groupe, trois doses répétées de G-CSF (dilué dans la Bovine Serum Albumin BSA à 1%) ont été injectées aux jours 1, 2 et 3 post-coïtum, tandis que dans le deuxième groupe, une injection unique de G-CSF a été réalisée le premier jour post-coïtum. Par ailleurs, des souris femelles CBA / J issues de chaque type de croisement ont reçu les mêmes schémas d'injection que précédemment décrits, mais réalisé avec uniquement de la BSA 1%, formant ainsi les deux groupes placebo injectés. Nous avons finalement formé deux groupes de contrôles noninjectés issus de chaque croisement.

4.3. Evaluation des Taux de Résorption et d'Implantation Embryonnaires

Les femelles issues des différents groupes ont été sacrifiées au 10eme jour de la gestation. Dans les cornes utérines, le nombre de sites d'implantation embryonnaire, ainsi que le nombre d'embryons résorbés ont été évalués pour chaque groupe. Le taux de résorption embryonnaire pour chaque femelle a été déterminé comme étant le rapport entre le nombre de sites d'implantation résorbés et le nombre total de sites d'implantation. Les taux de résorption et d'implantation embryonnaires ont été comparés entre les femelles de chaque croisement, pour différents schémas de supplémentation en G-CSF, d'injection de placebo, ou des contrôles non-injectés.

4.4. Article Original dédié aux effets du G-CSF sur les Modèles Murins

Un article de recherche original dédié à ce sujet et intitulé « Variations of Embryo Implantation and Embryo Resorption Rates after Systemic Supplementation by Granulocyte-Colony Stimulating Factor in an Abortion Prone and Control Murine Mating Combinations » a été soumis au « Journal of Reproductive Immunology », et est présenté dans les annexes de ce travail.

5. Discussion, Conclusion et Perspectives

5.1. Effets du G-CSF sur l'Endomètre Humain

Les gènes sélectionnés et considérés comme cibles potentielles du G-CSF dans l'endomètre présentent différentes fonctions impliquées dans l'implantation embryonnaire : facteurs de croissance, promoteur de migration cellulaire, molécules d'encrages, régulateurs de prolifération cellulaire.

En comparant les femmes fertiles aux patientes infertiles avec des antécédents de fausses couches répétées idiopathiques ou échecs répétés d'implantation embryonnaire idiopathiques, nous avons remarqué des variations importantes dans l'expression endométriale des différents gènes cibles préalablement sélectionnés.

Dans le modèle ex vivo, des variations d'expression des gènes cibles ont été observées aux plus fortes doses de stimulation par le G-CSF. Ces variations étaient spécifiques au G-CSF et n'ont pas eu lieu lorsque les mêmes expériences contrôles ont été réalisées avec le GM-CSF (Granulocyte-Macrophage-Colony Stimulating Factor).

Dans l'endomètre humain au cours de la fenêtre d'implantation, après avoir montré la différence d'expression de certains gènes cibles chez des patientes infertiles, et après avoir observé les variations d'expression de ces gènes cibles sous stimulation spécifique du G-CSF ex vivo, notre étude met en évidence le rôle essentiel du G-CSF au cours du processus implantatoire. Cette cytokine semble capable de moduler les gènes fondamentaux intervenant localement dans l'adhésion de l'embryon, la migration cellulaire, le remodelage tissulaire et l'angiogenèse, phénomènes nécessaires pour une implantation et une placentation réussies.

5.2. Effets du G-CSF sur le Modèle Murin

Le croisement murin femelle CBA / J x male DBA / 2 est un modèle de fausses couches spontanées a répétition, comparé au croisement fertile femelle CBA / J x male Balb / c. Les

mécanismes de résorption embryonnaire dans ce modèle ne sont pas liés au CMH et sont supposés être provoqués par la présence d'antigènes paternels provoquant une protection (par Balb / c) ou induisant un avortement (par DBA / 2). Ces antigènes seraient présents dans le plasma séminal et pourraient moduler localement l'environnement endométrial via des cellules T régulatrices et les cellules dendritiques. L'intervention du biosenseur endométrial est donc essentielle dans ce modèle murin.

Le schéma de supplémentation en G-CSF que nous avons décrit a été choisi pour cibler spécifiquement l'endomètre pré-implantatoire, en évitant des effets plus précoces sur l'ovulation ou des effets ultérieurs sur la croissance placentaire ou embryonnaire.

Dans ce modèle murin spécifique, les différentes variations que l'on observe sur les taux d'implantation ou de résorption embryonnaires témoignent d'une modulation du biosenseur endométrial par la supplémentation en G-CSF. L'effet endométrial de l'administration de G-CSF semble dépendre des doses et de la cinétique d'injection. Par ailleurs, lorsque l'on considère les effets induits par le G-CSF sur notre modèle murin fertile, cette supplémentation ne semble pas inoffensive.

5.3. Conclusion and Perspectives

La famille des CSFs est une illustration de la portée de l'immunologie de la reproduction. Ces cytokines et leurs récepteurs sont localisés le long de l'appareil reproducteur, et non seulement dans les cellules immunitaires. Grâce à divers effets locaux sur l'immuno-modulation ou l'immuno-trophicité, ils agissent sur les fonctions reproductives telles que l'ovulation, l'implantation embryonnaire ou le développement embryonnaire ou placentaire. Des études fondamentales sur les CSF, leur localisation, leurs variations physiologiques ou pathologiques, leurs diverses actions, ont généré des applications directes dans la médecine de la reproduction. Malgré leur intérêt indéniable en médecine de la reproduction, leurs indications précises ainsi que les mécanismes d'actions des CSFs ne sont pas encore élucidés, en particulier concernant les effets à long terme de leur utilisation sur les premiers stades du développement embryonnaire. De plus, une cinétique de supplémentation inadaptée (dose, voie d'administration) pourrait interférer avec un

environnement immunitaire endométrial propice et induire des défauts d'implantation et de développement embryonnaire.

Le G-CSF recombinant humain en particulier apparaît comme une thérapie innovante et prometteuse dans certains cas difficiles et non résolus d'échec de la reproduction, compte tenu de son action sur les mécanismes fondamentaux qui régulent le processus implantatoire. Toutefois, les indications médicales strictes de cette supplémentation dans le domaine de la reproduction et son mode d'administration ne sont pas encore établies.

Dans nos études, nous avons considéré l'endomètre decidualisé pré-implantatoire, sans la présence de l'embryon, et nous avons mise en évidence des dérégulations endométriales spécifiques. Ces observations impliquent que le diagnostic pré-conceptionnel et préventif de certaines dérégulations locales est possible.

L'application de nos observations au concept émergent mais fondamental de biosenseur endométrial permet de supposer que des thérapeutique ciblées pourraient améliorer la reproduction par le rétablissement pré-conceptionnel d'un endomètre réceptif. Les indications spécifiques d'une supplémentation en GCF en cas de troubles de la reproduction pourraient aussi être issues du diagnostic de dérégulations locales de certains de ses cibles endométriales.

L'optimisation de la réceptivité endométriale pourrait être d'un grand intérêt et faire partie des futures thérapies, non seulement spécifiquement dans la médecine de la reproduction, mais à plus long terme, en empêchant d'autres pathologies obstétricales qui découleraient d'une implantation embryonnaire initiale défectueuse.

