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Endometrial Factors and Blastocyst Implantation

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Introduction

Compaction of the morula is during embryo development, the first event of cellular differentiation. The most significant event occurring during compaction is the emergence of two distinct blastomere populations: those remaining in contact with the outside (zona pellucida) are destined to form the trophectodermal lineage (TE, the future placenta and its membranes), and those inside the embryo are destined to form the inner cell mass (ICM) and later the embryo proper. The TE is the foetal tissue actively involved in implantation. Implantation of the human blastocyst is divided in at least two major steps: *attachment* and *penetration*. From a biological point of view, these steps are completely different. Studying attachment implies the identification of molecules that will allow the TE of the blastocyst and the endometrial epithelial cells to make contact through their apical membranes. Studying penetration implies to understand the mechanisms by which trophoblastic cells invade the endometrium. Here we will limit ourselves to trophoblast invasion.

Cytotrophoblastic cells (CTB) are derived from the TE cells of the blastocyst and represent a heterogeneous population during early pregnancy. After initial attachment of the blastocyst to the uterine lining, mononuclear CTB that surround the embryonic disc fuse to form a syncytiotrophoblast (STB). These multinucleated, terminally differentiated giant cells invade the pseudo-decidualised endometrium. Once the placental villi are formed, some CTB of anchoring villi (that contact the uterine wall) acquire a transiently invasive phenotype and invade the decidualised endometrium. Meanwhile, the CTB of floating villi (in the extra-villous space bathed by maternal blood) remain attached to the villous basement membrane. Therefore, CTB follow one of two existing differentiation pathways:

- Villous CTB (vCTB), considered as stem cells, form a monolayer of polarised epithelial cells that proliferate and eventually differentiate by fusion to form the STB.
- Extra-villous CTB (evCTB), derived from vCTB, represent a population of non-polarised and invasive cells.

These motile and highly invasive evCTB invade the maternal uterus. Much like cancer cells that so successfully migrate and invade adjacent host tissues as they metastasise, evCTB physiologically invade the maternal decidua, the endometrial spiral arteries and even the proximal third of the myometrium. It is these cells that are used to mimic human blastocyst implantation because they retain their invasive phenotype in-vitro.

Blastocyst implantation is considered as an "action-reaction" process, where the endometrium reacts (adapts) to the invading embryo hence the title of this article. At the molecular level though, implantation resembles more to an "action-action" type of process since

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many regulators of embryo (trophoblast) invasion are of both endometrial and embryonic origin and act on both tissues. This mini-review is not meant to be exhaustive but wants to explore only some of these "action-action" regulatory mechanisms and the reader who wants a more complete picture of the regulators involved in blastocyst implantation is advised to look at recent reviews (1-5). We intend to illustrate here the complexity of the pathways that regulate trophoblast invasion by describing only two potential paracrine regulators: osteopontin and chemokines.

Effectors of trophoblast invasion

Extra-villous CTB, the motile and highly invasive trophoblastic cells, as well as tumour cells, are invasive because they secrete proteases capable of digesting the endometrial extra-cellular matrix (ECM). Serine proteases, cathepsins and matrix metalloproteinase (MMP) have been implicated in the invasive phenotype of tumours as well as CTB (6). Among the different proteases that the embryo (CTB) mobilises to dig its way into the maternal endometrium, the most important are with no doubt the MMP. MMP, also termed matrixins, represent a family of more than 20 human zinc-dependent endopeptidases, collectively capable of degrading all components of ECM. According to their substrate specificity and structure, members of the MMP gene family can be classified into 4 subgroups:

(1) Gelatinases (MMP-2 and MMP-9) digest collagen type IV (the major constituent of basement membranes) and denatured collagen (gelatine).

(2) Collagenases, (MMPs-1, 8, 13) digest collagen type I, II, III, VII and X. They are thus appropriately designed for digesting the collagen of the interstitium ECM.

(3) Stromelysins (MMPs-3, 7, 10, 11 and 12) have a relatively, broad substrate specificity and digest collagen type IV, V, VII, as well as laminin, fibronectin, elastine, proteoglycans and gelatine.

(4) Membrane MMP (MMPs-14, 15, 16) cleave proMMP-2 and allow activation of MMP-2 at the cell surface of the invasive front.

Most MMPs are secreted as inactive pro-enzymes (zymogens) that become activated in the extra-cellular compartments with the exception of MMP-11 and MT-MMPs. Activation occurs by dissociation of a Zn^{2+}-Cys bond that leads to the loss of a pro-peptide. Several enzymes are capable of activating the pro-matrixins, the most prominent of them being plasmin (6). The activity of MMP in the extra-cellular space is specifically inhibited by tissue inhibitor of MMP (TIMP), which binds to the highly conserved zinc-binding site of active MMP at molar equivalence. The TIMP gene family consists of four structurally related members, TIMP-1, -2, -3 and -4 (7, 8 for reviews).

As recently observed, substrates for MMP are not limited anymore to ECM glycoproteins and the different collagens but comprise also several classes of proteins (9) such as cell surface molecules, adhesion molecules, mediators of apoptosis, receptors, chemokines, cytokines, growth factors, proteases, intercellular junction proteins, and structural molecules. All these pathways are particularly important for blastocyst implantation. MMP are thus not only the tools that digest the ECM to make space for the embryo but they are instrumental modulators of the acquisition of a physiological or pathological invasive phenotype.

In vitro, CTB invade a reconstituted basement membrane (Matrigel); behaving thus, like metastatic cells. This invasive behaviour is due to the ability of CTB to secrete MMP, since TIMP inhibits their invasiveness (10). Several studies have localised MMP proteins and mRNA in human trophoblast and cultured CTB secrete MMP (11). The fact that CTB from early pregnancy are more invasive and secrete more MMP than CTB isolated from the term

placenta is taken as evidence that these enzymes are regulated throughout pregnancy (12). One admits that CTB behave like aggressive tumour cells and secrete MMP from a very early stage in their development, since an 8-cells human embryo produces MMP (13).

The biological properties of MMP and TIMP increase the difficulty for us to understand how regulators of MMP will affect cell invasion since these regulators can act at different levels such as: MMP and TIMP gene transcription, post-translational modifications, activation of the zymogen and availability of the inhibitors. Based on quite a number of observations (14, 15 as examples) the decidualised endometrium is considered as the prime source of regulators essentially limiting embryo invasion (the action-reaction concept). But, trophoblast exerts quite a significant impact on the maternal endometrium (the reaction-reaction concept). Indeed, Popovici et al (16) using gene expression profiling, showed that in a co culture model of human trophoblast and endometrium, expression of an array of endometrial genes (including some MMP) were modified by the close vicinity of trophoblast.

Is osteopontin (opn) a key player at the foeto-maternal interface?

Osteopontin (OPN), also known as secreted phosphoprotein 1 (SPP1), bone sialoprotein I (BSP-1), early T-lymphocyte activation (ETA-1), is a glycoprotein that was first identified in osteoblasts. OPN is a secreted extracellular structural protein and therefore an organic component of bone. It is a heavily glycosylated 44kDa peptide that contains an arginine-glycine-aspartic acid (RGD) motif. This motif is recognised by adhesion molecules, such as integrins and particularly the aVb3 integrin considered as the OPN receptor. OPN has multiple functions: it has a role in cell adhesion, chemotaxis, prevention of apoptosis, invasion, migration and anchorage-independent growth of tumour cells. OPN has also a role in the regulation of cell signalling pathways that lead to neoplasia and malignant transformation. An elevated expression of OPN has been observed in a variety of cancers and has been linked with tumour metastasis and with a poor prognosis for the patient (see 17,18 for reviews).

OPN RNA and protein are expressed by human trophoblast throughout pregnancy. It was observed by immunohistochemistry that this peptide is localised in CTB but not in STB (19) and that it is strongly expressed at the invasive front on the evCTB of the placental bed (20). In primary cultures of CTB, OPN RNA disappears gradually as the cells fuse to form a non-invasive syncytium (19) and OPN treated first trimester CTB were more invasive than control CTB in the matrigel invasion assay (20). OPN must thus be considered as an autocrine regulator of trophoblast invasion.

There is much more to this concept. Indeed, OPN RNA and protein were also localised in early pregnancy decidua (21) and in normal endometrium throughout the menstrual cycle (22) where OPN expression was maximal in the mid secretory phase of the cycle thus during the implantation window at the time of maximal endometrial receptivity (22). Interestingly, the aVb3 integrin, the OPN receptor is not only detectable on the surface of first trimester CTB and STB (23) but also on endometrial epithelial cells of the uterine glands and particularly on the epithelium lining the endometrial cavity (21) at the time of maximal endometrial receptivity.

We are now in the situation where both receptors and ligands are concomitantly present on maternal and foetal tissues at this crucial time of implantation. These observations tend to suggest that besides its role as an autocrine regulator of trophoblast invasion, OPN is also a paracrine regulator. Furthermore, through an OPN-aVb3 integrin interaction, it could play a role in anchoring trophoblast to the maternal endometrium helping to immobilise the embryo on the endometrial lining.

It is not yet the end of the "OPN story". Expression of OPN is often co-localized with

members of the MMP family during tissue remodelling and tumorigenesis suggesting that in vivo a significant portion of OPN may be subject to proteolytic modifications in the extracellular milieu. Indeed, it was shown that OPN is a substrate for two MMP: MMP-3 (stromelysin-1) and MMP-7 (matrilysin). Three cleavage sites were identified for MMP-3 in human OPN, and two of those sites are also cleaved by MMP-7 (24). It is not really a surprise that an ECM glycoprotein such as OPN is cleaved by MMP, but what is relevant here is that some of the cleavage products are biologically more potent than the full length OPN (25) whereas others have lost their activity. This indicates that MMP besides being instrumental in trophoblast invasion (they digest the ECM), they also modulate the activity of OPN. Thus at the foeto-maternal interface OPN mediated embryo adhesion, trophoblast invasion and trophoblast anchoring are most probably modulated by the availability and activity of endometrial and trophoblastic MMP.

As indicated above, the regulation of MMP expression and secretion is complex because it can occur at different levels: gene expression, post-translational modifications, zymogen activation and active enzyme inhibition through TIMP. In the context of the present discussion it is interesting to know that in a metastatic murine mammary cancer cell line, OPN mediates transcriptional activation of its own gene, of MMP-2 (gelatinase-A) and of urokinase (uPA, the enzyme that cleaves plasminogen into plasmin allowing in vivo activation of MMP). OPN acts through binding to its surface receptor (26). Activation of an integrin-linked kinase by OPN-aVb3 integrin complexes results in binding of activator protein-1 (AP-1) to MMP-2 and uPA promoter increasing thereby transcription of these genes. Recently, another transduction mechanism was described in a human breast cancer cell line where OPN induced MMP-2 and uPA gene transcription through activation of another transcription factor: NFkB (27). We do not know so far if these activities of OPN are also true at the foeto-maternal interface, but we know that all the players are there and that NFkB and AP1 are not only trans-activators of MMP-2 but of several other MMP produced by the embryo and the mother (28).

The "OPN story" illustrates the incredibly high degree of complexity of interacting pathways that can potentially regulate blastocyst implantation in the human. It definitively condemns the simplistic image of paracrine (from the decidua) or autocrine (from the trophoblast) regulators that stimulate or inhibit embryonic enzymes that digest endometrial ECM.

Are chemokines key players at the foeto-maternal interface?

Humans have a haemochorial type of implantation indicating that the very invasive foetal trophoblast comes in direct contact with maternal blood. Haemochorial mammals (mice, rats and guinea pigs) were thus used as models to study human implantation. If these models allowed quite a number of interesting observations (among them the description of the window of implantation) they all have their limitation and no one can entirely mimic the human situation. This prompted researchers to develop in-vitro cell models that could reproduce the important steps of the implantation process such as adhesion, attachment and invasion. In 2003, Olga Genbacev and her group (29) proposed that at the morphological level (at least), leukocyte extravasation bears quite a number of features in common with embryo implantation and consequently, the initial steps of implantation could make use of the same molecular mediators as leukocyte extravasation. In the vasculature, leukocytes bind to endothelium despite the presence of a strong blood flow. This interaction is governed by carbohydrate-binding proteins (selectins) that immobilise the cells on the vascular wall allowing them to transmigrate into the underlying tissue. This original comparison allowed Genbacev's group to show that on the

maternal side, human uterine epithelial cells up-regulate selectin receptors during the window of implantation and on the foetal side, CTB express L-selectin. This ligand-receptor system was shown to be functional, because beads coated with selectin ligands bound CTB (29).

The analogy between leukocyte extravasation and blastocyst implantation can in fact be taken somewhat further. Indeed leukocytes do not cross the vessel wall anywhere but only at places where they are needed thus where inflammation occurs. Leukocytes know where to escape the circulation because they are attracted by chemo attractants: the chemokines. Chemokines are small (about 10 kDa) structurally related chemotactic cytokines that regulate cell trafficking of various types of leukocytes through interactions with a subset of seven-transmembrane, G protein–coupled receptors (30-32 for reviews). About 50 chemokines and 20 receptors have now been identified in humans (Table I) and a novel classification proposed for both partners (30). Chemokines and their receptors are divided in two families based on structural criteria: chemokines have at least four cysteines in a conserved positions. The CXCL chemokines have two cysteines (C) in the N-terminus that are separated by a single variable (X) amino acid whereas the CCL chemokines have the two cysteines without any amino acid between them. A capital L is added to identify a ligand by comparison with the R added to identify the receptor. Most chemokines bind to cell-surface receptors or to connective-tissue components such as glycosaminoglycans. Chemokines mainly act on neutrophils, monocytes, lymphocytes, and eosinophils and play a pivotal role in host defence mechanisms. Furthermore, the chemokines exert a wide range of effects in many different cell types beyond the immune system, and CTB and endometrial cells are no exceptions.

Chemokine receptors belong to the super family of G-protein-coupled receptors that have seven sequences of amino acids spanning the plasma membrane, an extracellular N-terminus and an intracellular C-terminal tail. These receptors signal the cell about the presence of chemokine gradients in the extracellular environment. They are named CXCR or CCR depending on the structure of their ligand (CXCL or CCL). Despite the fact that several chemokines bind the same receptor, there is a certain degree of specificity (Table I).

The Spanish group of Carlos Simon was among the first to investigate the chemokines at the foeto-maternal interface (33, 34). They showed that CXCL8 (interleukin-8, IL-8), CCL1 (Monocyte chemotactic protein-1, MCP-1) and CCL5 (regulated upon activation normal T cells **e**xpressed and **s**ecreted, RANTES) are potent chemo attractants expressed and secreted by human endometrium during the secretory phase. CXCL-8 and CCL1 are of epithelial origin, whereas CCL5 is produced by endometrial stromal cells (33). Chemokine receptors such as CCR2, CCR5, CXCR1 and CXCR4 were also investigated and shown to be present in the endometrium. If the chemokines CXCL8, CCL1 and CCL5 are not secreted by human blastocyst, the receptors CCR2 is expressed by the inner cell mass, whereas CCR5 is expressed by the trophectodermal cells (33). The authors suggest in their article that chemokines and their receptors are involved in the implantation process. The impact of endometrial chemokines in implantation and placentation has been reviewed a few years later by the Australian group of Lois Salamonsen (35, 36).

Table I is only an overview meant to focus attention of the reader on this relatively new field in reproductive biology and it summarises our present knowledge in the field. Despite the fact that this table does not take into consideration the precise cellular origin of the chemokine or its receptor (endometrial epithelial or stromal cells or granular lymphocytes, T cells or macrophages and cyto- or syncytiotrophoblast or foetal fibroblasts),

Table I. Localisation of the chemokines and their receptors at the foeto-maternal interface in humans (systematic PubMed search with receptor or ligand name followed by "and trophoblast" or "and endometrium").

Present in		Receptors	Ligands	*Present in*	
Endometrium Decidua	*Embryo Trophoblast*			*Endometrium Decidua*	*Embryo Trophoblast*
pos	pos	CXCR1	CXCL6	pos	pos
pos	pos	CXCR1	CXCL8	pos	pos
pos	pos	CXCR2	CXCL1	pos	
	pos	CXCR2	CXCL2	pos	
	pos	CXCR2	CXCL3	pos	
	pos	CXCR2	CXCL5		pos
	pos	CXCR2	CXCL6	pos	pos
	pos	CXCR2	CXCL7		
	pos	CXCR2	CXCL8	pos	pos
pos	pos	CXCR3	CXCL9	pos	neg
pos	pos	CXCR3	CXCL10	pos	neg
pos	pos	CXCR3	CXCL11	pos	neg
pos	pos	CXCR4	CXCL12	neg	pos
pos	pos	CXCR5	CXCL13	pos	
		CXCR6	CXCL16		pos
	pos	CXCR7	CXCL11		
	pos	CXCR7	CXCL12	neg	pos
pos	pos	CCR1	CCL3		
pos	pos	CCR1	CCL5	pos	
pos	pos	CCR1	CCL7	pos	pos
pos	pos	CCR1	CCL8	pos	
pos	pos	CCR1	CCL13		
pos	pos	CCR1	CCL14	pos	pos
pos	pos	CCR1	CCL15		
pos	pos	CCR1	CCL16	pos	
pos	pos	CCR1	CCL23		
pos	pos	CCR2	CCL2	pos	pos
pos	pos	CCR2	CCL7	pos	pos
pos	pos	CCR2	CCL8	pos	
pos	pos	CCR2	CCL13		
pos	pos	CCR2	CCL16	pos	
pos	pos	CCR3	CCL5	pos	
pos	pos	CCR3	CCL7	pos	pos
pos	pos	CCR3	CCL8	pos	
pos	pos	CCR3	CCL11	pos	
pos	pos	CCR3	CCL13		
pos	pos	CCR3	CCL15		
pos	pos	CCR3	CCL24		
pos	pos	CCR3	CCL26		
pos	pos	CCR3	CCL28		
pos	pos	CCR4	CCL17	pos	
pos	pos	CCR4	CCL22	pos	
pos	pos	CCR5	CCL3	pos	pos
pos	pos	CCR5	CCL4	pos	pos
pos	pos	CCR5	CCL5	pos	
pos	pos	CCR5	CCL8	pos	
	pos	CCR6	CCL20	pos	
	pos	CCR7	CCL19	pos	
	pos	CCR7	CCL21	pos	
pos		CCR8	CCL1		
		CCR9	CCL25		
	pos	CCR10	CCL27		
	pos	CCR10	CCL28		

Pos: positive, presence of ligand or receptor message or protein in tissue or cells including cell lines. **neg**: negative, absence of ligand or receptor message or protein in tissue or cells including cell lines. **empty cell**: no data found.

one can still see that ligands and receptors are on both sides of the foeto-maternal interface implying thus a bi-directional communication another example of a reaction-reaction process. Table 1 illustrates also the degree of complexity of some regulators that might govern the embryo-endometrial summit.

There are a few studies describing in-vitro effects of chemokines in relation to embryo implantation or trophoblast invasion. These studies are all based on the hypothesis that endometrial chemokines regulate trophoblast invasion. It was observed that endometrial cell conditioned medium or CCL4 and CCL14 but not CCL7 significantly stimulate migration of an extra villous trophoblast cell line (37) and up-regulate a number of trophoblastic genes including OPN and MMP-12 (38). CXCL8 (IL-8) is produced by endometrial epithelial and stromal cells under the stimulus of trophoblastic interleukin-1, and significantly increases the migration of primary CTB (39) indicating that trophoblast and endometrium cooperate to regulate CTB (embryo?) invasion. CXCL12 is produced by trophoblast but not by decidual cells in-vitro and simulates CTB invasion and the activities of MMP-2, -9 (40) and MT1-MMP (41). This clearly implicates CXCL12 in the autocrine regulatory loop of trophoblastic invasion.

Besides endometrium and trophoblast, chemokines from other sources seem also to affect implantation/trophoblast invasion. Indeed, Japanese colleagues based at the University of Kyoto showed that chemokines derived from (maternal) blood platelets, increase invasion and MMP-2, -9 secretion by human evCTB (42) and that human chorionic gonadotrophin (hCG) stimulates CXCL8 (IL-8) secretion by peripheral blood monocytes derived from non-pregnant women (43). These important observations suggest that near the implantation site, in the spiral arteries of the endometrium (physiologically invaded by CTB), maternal blood contains chemokines derived from platelets or monocytes that are capable to direct implantation and placentation.

Yet another level of complexity has to be considered when looking at the possible impact of chemokines on embryo implantation. Chemokines not only stimulate invasion and MMP secretion (42, 44) but MMP cleave and inactivate CCL7 (45), CCL12, CCL8, CCL13 (46) and CXCL12 (47). Moreover cleavage of CCL7 by MMP-2 occurs through binding of the chemokine to the hemopexin-like domain of MMP-2 generating a potent receptor antagonist (CCR1, CCR2 and CCR3, 45). The truncated products of CCL8 and CCL13, like CCL7, are also potent antagonists of their cognate CC chemokine receptors as evidenced by a cell migration assays (46). MMP-mediated proteolysis of chemokines is a potent regulatory mechanism (recently reviewed in 48) that profoundly modifies the activity of chemokines by either inactivating, antagonising or even potentiating the biological activity of these compounds. It must however by remembered here that instead of being presented to proteases as soluble proteins most chemokines are immobilised in the ECM (bound to glucoseaminoglycans), and thus the modulatory activity that MMP can exert on chemokines will be translated into modifications of an endometrial chemokine gradient.

Similarly to what we have said above for OPN, chemokines and their receptors are expressed on both sides of the foeto-maternal interface, they regulate embryo invasion by modulating cell migration and MMP secretion but their activity is in turn modulated by MMP.

Conclusion

The action-reaction view of the implantation process can no longer be valid, maternal and foetal tissues interact at a very particular moment programmed by maternal hormones. Regulators and effectors act and are produced on both sides of the fence, their activity can be modulated by enzymes (MMP) that both tissues produce. Other maternal cells than the ones

immediately involved in implantation are also capable of modulating the embryo-endometrium encounter by using similar regulators as those produced locally in the uterus. Pathways of implantation/invasion regulators such as OPN or chemokines but also cytokines, growth factors, hormones and others are intricately mixed at several key points allowing for an important degree of biological redundancy, a necessary security for such an important process as embryo implantation. Understanding this degree of complexity is a huge task that no one has accomplished yet. We have the alphabet (OPN, MMP, CCL, CXC etc) we can read the words but we need a Rosetta stone to understand the meaning.

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