Molecular interactions between embryo and uterus in the adhesion phase of human implantation

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Molecular interactions at the embryo–maternal interface at the time of implantation is an exciting field demanding a wide effort in order to understand the crucial process of embryonic implantation. The objective of the present work is to demonstrate the existence of a specific communication pathway (at the molecular level) between embryo and endometrium in the adhesion phase of human embryonic implantation. This pathway of molecular interactions is apparently initiated by the endometrium in the presence of an implanting blastocyst. It is mediated through the embryonic interleukin (IL)-1α + IL-1β, and the target is the endometrial epithelial β3 integrin subunit. If the relevance of β3 is accepted as a marker of uterine receptivity, these observations may imply that the normal hormonally-regulated human endometrium is the trigger of molecular events preparing the blastocyst to efficiently communicate and regulate endometrial adhesion molecules in order to implant.

Key words: adhesion molecules; cytokines; embryo; endometrium; implantation

Introduction

Implantation is a progressive process in which the embryo has to appose and attach itself to the maternal endometrium and invade into it. The most intriguing aspect of implantation is that it involves two outstanding players: the maternal endometrium and the embryo. Communication between them, and the reciprocal effect on each other is an exciting and as yet unsolved paradigm in reproductive medicine. Once it begins, it has been estimated that clinical implantation in the human is efficient in no more than 30% of cases (Miller et al., 1980). The responsibility of this low implantation efficiency has to be shared between the embryo, e.g. 30% of blastocysts are morphologically abnormal at the time of implantation in vivo (Hertig et al., 1952) and a defective embryonic–endometrial dialogue, e.g. 30% of early pregnancy losses occur before the expected time of menstruation (Wilcox et al., 1988). An even higher amount of early embryonic
A general hypothesis has been postulated (Figure 1). Cytokines such as leukaemia inhibitory factor (LIF) and interleukin-1 (IL-1) and their specific receptors, properly distributed throughout endometrium and embryo and adequately controlled at the endocrine and paracrine/autocrine (cytokine and growth factor) levels, may start the mutual recognition of implanting blastocyst and endometrium (Simón et al., 1996a). Cytokines and growth factors may also serve as the link in the regulation of molecules that provide the physical contact between embryo and uterus, referred to as adhesion molecules (Simón et al., 1996a). Since redundancy is one premise to assure the effectiveness of crucial biological processes, many communication possibilities between embryo and endometrium must exist, but unique systems could also be at play.

The endometrium is an active, versatile and hormonally regulated ‘organ’ which plays a major role in the process of implantation and in maintaining a viable pregnancy. It has traditionally been perceived as a passive counterpart. Recent evidence, however, suggests that the maternal endometrium has to be prepared, in that it is ‘receptive’ to an implanting blastocyst only within a ‘window of implantation’, which is temporally and spatially restricted.

The crucial role of steroid hormones to prepare and drive the endometrium for successful embryonic implantation is beyond any doubt. However, it is clear that steroid hormones are not final effectors, rather they may initiate a ‘downstream’ cascade of molecular events through local paracrine/autocrine molecules.
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which account for the intimate mechanisms of receptivity. We are just beginning to understand the expression, regulation, and mode of action of several cytokines, growth factors, and adhesion molecules during the implantation window (for review see Giudice, 1994; Lessey and Castelbaum, 1995; Tabibzadeh et al., 1995a,b). The mouse model has provided insight into the relevance of some molecules relevant to endometrial preparation. These effector molecules include members of the epidermal growth factor (EGF) family, LIF and IL-1.

A newly discovered member of the EGF family, heparin-binding EGF (HP-EGF) is expressed in uterine luminal epithelium at the site of implantation before any other sign of blastocyst attachment and disappears when implantation is delayed by administration of progesterone (Das et al., 1994). During pregnancy, LIF is expressed in mouse endometrial epithelium on the fourth day in the presence or absence (i.e. in pseudopregnant animals) of an embryo (Stewart, 1994). In fact, blastocyst implantation depends on the uterine expression of LIF in mice (Stewart et al., 1992), demonstrated by the transgenic mouse model. On the other hand, blockade of endometrial interleukin-1 receptor type I (IL-1R tl) by its natural antagonist interleukin-1 receptor antagonist (IL-1ra) prevents implantation in mice (Simón et al., 1994a) by a direct effect on the endometrial epithelium. (Simón et al., 1998) In humans, it has been demonstrated the capability of the endometrium to produce LIF in vivo has been demonstrated (Laird et al., 1997); however, the precise role for these cytokines in preparing the human endometrium for implantation remains undefined.

In this work we shall concentrate on the possible endometrial–embryonic dialogue implicating the IL-1 system in humans. The dynamics of the complete IL-1 system, IL-1β (Kauma et al., 1990; Tabibzadeh and Sun, 1992; Simón et al., 1993a), IL-1R tl (Tabibzadeh et al., 1990; Simón et al., 1993a,b) and IL-1ra (Tabibzadeh and Sun, 1992; Sahakian et al., 1993; Simón et al., 1995) have been clarified in the endometrium, endometrial fluid (Simón et al., 1996b), human embryos (De los Santos et al., 1996) and at the maternal–embryonic interface (Paulesu et al., 1991; Hu et al., 1992; Librach et al., 1994; Simón et al., 1994c). These morphological data are in agreement with the demonstrated stimulatory effect of IL-1 on human chorionic gonadotrophin (HCG) release from human first trimester trophoblast cells in culture (Mashuhiro et al., 1991; Steelee et al., 1992).

The aim of this paper is to analyse one specific pathway of molecular interactions between embryo and uterus in the adhesion phase of human implantation. Based on previous and actual studies, our hypothesis (Figure 2) is that the embryo secretes the complete IL-1 system (IL-1α, IL-1β/IL-1ra) when entering the endometrial cavity in response to the presence of endometrial epithelial cells (EEC) (De los Santos et al., 1996). In turn, the blastocyst acts on the maternal EEC by up-regulating the β3 integrin subunit and this activation is triggered by the binding and activation of embryonic IL-1α + IL-1β to the endometrial epithelial IL-1R tl (Simón et al., 1998). This IL-1-induced endometrial β3 up-regulation is functionally relevant because it increases the ability of the blastocyst to adhere to the EEC.
Figure 2. Model for adhesion of the blastocyst to endometrial epithelium during human implantation. In response to an unknown endometrial factor, the human embryo secretes the complete interleukin-1 (IL-1) system (IL-1α, IL-1β, IL-1ra) which is constitutively present but not secreted, as demonstrated by the selective release in the presence of endometrial epithelial cells (EEC). The binding and activation of embryonic IL-1α + IL-1β to the endometrial epithelium induces an up-regulation of β3 subunit which is morphologically present and functionally relevant because it increases the ability of the blastocyst to adhere to the EEC monolayer.

Material and methods

Experimental design

To investigate the effect of endometrial cells on the regulation and secretion of the interleukin-1 system by the human embryos we first analysed the immunohistochemical presence of IL-1β, IL-1ra and the common receptor IL-1R t1 in human oocytes and embryos. Secondly, the IL-1 embryonic secretion pattern was evaluated in the conditioned media of embryos cultured under different conditions. Thirdly, the possibility that an embryo–endometrial interaction was involved in the release of these cytokines by the human embryo was investigated by co-culturing individual human embryos with human endometrial stromal cells (ESC) or with human endometrial epithelial cells (EEC).

To investigate the effect of single human embryos in regulating β3, α4 and α1 integrins on human endometrial epithelial cells, we have developed a clinical program in which embryos are co-cultured with endometrial epithelial cells (EEC) until the blastocyst stage and transferred back to the mother. EEC wells were divided according to the embryonic status: EEC with embryos that reached the blastocyst stage, EEC with arrested embryos and EEC without embryos. Under these conditions, we have morphologically localized these molecules by immunocytochemistry. Second, to quantify the embryonic regulatory effect on EEC monolayers, flow cytometry of β3, α4 and α1 integrins was performed. Third, the possibility that the embryonic IL-1 system was involved in the
endometrial $\beta_3$ up-regulation was investigated by neutralizing experiments and further confirmed by scanning electron microscopy (SEM) and dose response experiments. Finally, the functional significance of the endometrial $\beta_3$ up-regulation induced by the embryo was studied using a mouse embryonic adhesion assay.

**Clinical in-vitro fertilization (IVF) protocol**

The ovarian stimulation protocol using gonadotrophin-releasing hormone (GnRH) analogues and gonadotrophins has been previously described (Pellicer et al., 1989). Only type I and type II embryos were used for the present study corresponding to embryos with regular blastomeres without fragmentation and embryos with regular blastomeres with $<25\%$ of fragmentation respectively.

**Immunohistochemical staining**

The immunostaining procedure was performed on human oocytes and embryos by an avidin–biotin alkaline phosphatase technique as described (De los Santos et al., 1996). Primary antibodies were monoclonal mouse anti-human IL-1R $\alpha$ antibody, monoclonal mouse anti-human IL-1$\beta$ antibody at 20 $\mu$g/ml each, polyclonal rabbit anti-human IL-1$\alpha$ antibody at 20 $\mu$g/ml (all from Genzyme Corporation, Cambridge, MA, USA).

Endometrial epithelial cells grown on 8-chamber tissue culture slides (Lab Tek; Miles Scientific, Naperville, IL, USA) were analysed by indirect immunofluorescence as reported (Simon et al., 1998) using as primary antibodies: TS2/7 mouse anti-human $\alpha_1$ (1:10000 dilution), B-5410 mouse anti-human $\alpha_4$ (1:3000 dilution), AP3 mouse anti-human $\beta_3$ (1:1500 dilution) each for 60 min at room temperature.

**Embryo culture**

Human embryos at different developmental stages were cultured under different conditions. Between three and four human embryos were cultured under oil in 100 $\mu$l drops of Ham's F-10 (Gibco BRL, Paisley, Scotland) + 4 mg/ml bovine serum albumin (BSA; Pentex, Miles Diagnostic Division, Kankakee, USA) ($n = 33$) or Ménézé B2 (Lab CCD, Paris, France) ($n = 18$) or in wells with 1 ml of Ménézé B2 culture media ($n = 8$). Embryo conditioned media (CM) was removed every 24 h for IL-1$\alpha$, IL-1$\beta$ and IL-1$\alpha$ measurements.

**Embryo co-culture**

Endometrial samples obtained from secretory endometrium were minced into small pieces $<$1 mm, subjected to mild collagenase digestion. Endometrial stromal cells (ESC) and endometrial epithelial cells (EEC) were isolated as previously described (Simón et al., 1994b). For embryo co-culture, individual
human embryos were co-cultured with experimental EEC and ESC, \( n = 23 \) and \( n = 4 \) respectively) and with EEC conditioned media \( n = 9 \) for 5 days (De los Santos et al., 1996).

In a second set of experiments, individual human embryos were co-cultured with experimental EEC for 5 days (Simón et al., 1998). Embryos achieving the blastocyst stage were transferred back to the mother. After embryo transfer, EEC wells were divided according to the embryonic status reached: EEC with embryos that achieved the blastocyst stage, EEC with arrested embryos and EEC without embryos.

**Flow cytometry**

For FC experiments, EEC monolayers were detached by treatment with HBS 1 mM EDTA/trypsin EDTA (1/1), processed and incubated with same antibodies for \( \beta_3, \alpha_4 \) and \( \alpha_1 \) as described elsewhere (Simón et al., 1998). Data were expressed as the percentage of stained cells.

**Neutralizing experiments**

Monolayers of EEC were cultured for 24 h in a pool of conditioned media from co-cultured blastocysts in the absence \( (n = 8) \) or presence of saturating concentrations of anti-hIL-1\( \beta \) (5 \( \mu \)g/500 ml) + anti-hIL-1\( \alpha \) (500 \( \mu \)g/500 \( \mu \)l) \( (n = 4) \), with saturating doses of anti-hIL-1ra (1mg/500 ml) \( (n = 4) \), with recombinant human IL-1r\( \alpha \) (10 \( \mu \)g/500 \( \mu \)l) \( (n = 3) \) or with blocking doses of anti-LIF (250 \( \mu \)g/ 500 \( \mu \)l) \( (n = 4) \) (Simón et al., 1998).

**Dose–response experiments**

EEC monolayers were cultured for 48 h in the presence of increasing concentrations of IL-1\( \alpha \), IL-1\( \beta \) and IL-1\( \alpha \) + IL-1\( \beta \) of 0, 1, 10, 100 and 1000 pg/ml (Simón et al., 1998).

**Enzyme-linked immunosorbent assay (ELISA)**

IL-1\( \alpha \), IL-1\( \beta \) and IL-1ra concentrations were measured in the conditioned media from dose -response experiments using a kit from R&D Systems (Minneapolis, MN, USA) (De los Santos et al., 1996; Simón et al., 1998).

**Mouse embryonic adhesion assay**

To analyse the adhesion enhancement a mouse model described by (Austgulen et al., 1995) and reported by our group (Simón et al., 1998) was employed.

**Statistical analyses**

Percentage of cells stained were expressed as mean ± SEM. For statistical comparison among groups, \( \chi^2 \) or analysis of variance (ANOVA) followed by
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Table I. HSCORE of oocytes and embryos analysed for the immunoreactive presence of interleukin (IL)-1β, IL-1ra and IL-1Rt-I

<table>
<thead>
<tr>
<th></th>
<th>Oocyte (n = 44)</th>
<th>2–3 cell (n = 16)</th>
<th>4-cell (n = 15)</th>
<th>6-cell (n = 15)</th>
<th>8-cell (n = 10)</th>
<th>&gt;10-cell (n = 3)</th>
<th>Morula (n = 3)</th>
<th>Blastocyst (n = 12)</th>
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<tr>
<td>IL-1β</td>
<td>2.77</td>
<td>2.37</td>
<td>2.99</td>
<td>3.5</td>
<td>2.15</td>
<td>3.01</td>
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<tr>
<td>IL-1Rt-I</td>
<td>2.52</td>
<td>3.00</td>
<td>3.00</td>
<td>3.17</td>
<td>1.80</td>
<td>1.50</td>
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<td>1.98</td>
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<td>(n = 40)</td>
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<tr>
<td>IL-1ra</td>
<td>3.67</td>
<td>3.25</td>
<td>3.83</td>
<td>-2.75</td>
<td>1.75</td>
<td>4.00</td>
<td>2.34</td>
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<td>(n = 34)</td>
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aData are not significantly different.

Fisher’s PLSD test were applied; P ≤ 0.05 was considered to be statistically significant.

Results

Embyronic response to the maternal endometrium

To investigate the potential role of the IL-1 system in the human embryo, we first localized the presence of immunoreactive IL-1β, IL-1ra and the common receptor IL-1R tI during the preimplantation period. Immunostaining for IL-1β, IL-ra and IL-1Rt-I was confirmed in oocytes and embryos in all developmental stages analysed. During embryonic development, IL-1β, IL-1ra and IL-R tI staining were confined to the cytoplasm and plasma membrane with no statistical differences among different developmental stages (Table I).

IL-1α and IL-1β were absent in conditioned media (CM) of embryos cultured either in 100 μl drops under oil of Ham’s F-10 + 4 mg/ml BSA, Ménézo B2 or 1 ml of Ménézo B2 at all developmental stages analysed. The only exception, detectable levels of these cytokines were found in three out of five conditioned media obtained 24 h after insemination of oocytes.

Since the immunoreactive protein was present, we focused our research on the possibility that human endometrial factors were involved in the release of these cytokines by the human embryo. IL-1α, IL-1β and IL-1ra were absent in CM of embryos co-cultured with ESC and their controls. However, when single human embryos were co-cultured with endometrial epithelial cells (EEC), two different populations of embryos were observed: IL-1 producers, described as embryos producing IL-1α and/or IL-1β, and IL-1 non-producers, from which neither IL-1α nor IL-1β were detected. To confirm the positive effect of endometrial epithelial cells on the embryonic IL-1 system secretion, human embryos were cultured with endometrial epithelial cells conditioned media alone showing a similar pattern, as described above. We further obtained the IL-1α-IL-1β and IL-1ra secretion patterns from the IL-1 producer population of single human embryos co-cultured with EEC during embryonic development (Figure
3). The concentrations (mean ± SE) of these cytokines were 1.5 ± 0.4, 0.48 ± 0.21 and 137.1 ± 42.9 for IL-1α, IL-1β and IL-1ra respectively. Values expressed in Figure 3 were obtained after subtraction of the levels from controls performed each day from the values obtained in the co-culture. No differences were found in IL-1α-IL-1β and IL-1ra levels during embryonic development. However, the ratio IL-1α-IL-1β/IL-1ra at the blastocyst stage was significantly lower compared to the ratios at the 4-cell and 6-cell stages.

**Embryonic impact into the maternal endometrial epithelium**

To investigate the potential embryonic regulation of endometrial integrins, we first localized the presence of immunoreactive β3, α4 and α3 in cultured human EEC. After embryo transfer, EEC wells were divided according to the embryonic status reached: EEC with embryos that achieved the blastocyst stage, EEC with arrested embryos and EEC without embryos. Immunostaining for β3 was positive in plasma membrane of EEC with increased intensity in wells from embryos that reached blastocyst stage compared to those EEC wells from arrested embryos. Flow cytometry (FC) showed a mean percentage of β3 stained cells of 24.1 ± 5.7 in EEC co-cultured with embryos that achieved the blastocyst stage (n = 13) versus 9.5 ± 1.6 (P < 0.05) in EEC cultured with arrested embryos (n = 12). Immunostaining for α1 and α4 integrins was negative in EEC monolayers studied, regardless of the presence or absence of embryos, and these findings were confirmed by flow cytometry. The possibility that the embryonic IL-1 system was involved in the endometrial β3 up-regulation was investigated by neutralizing experiments demonstrating a significant inhibition of β3 stained cells when EEC monolayers were cultured in the presence of EEC/blastocyst-conditioned media with (n = 4) versus without (n = 8) anti-human IL-1α + IL-1β (1.65 versus 14.6%; P < 0.05) (Figure 4).

Scanning electron microscopy (SEM) further demonstrated that EEC cultured with conditioned media from co-cultured blastocyst (Figure 5B) were healthy. Their shape was more rounded when compared with the control EEC cultured without embryos (Figure 5A). Also, retraction fibres (a sign of cell migration) and specifications of the plasma membrane such as short-stubby and long-hairy microvilli (Figure 5E) were much more abundant when compared with controls (Figure 5D). Strikingly, EEC in contact with conditioned media from co-cultured blastocysts developed a bulging of the membranes resembling pinopods (Figure 5H), a feature not found in control EEC (Figure 5G). When the IL-1 system was blocked in those EEC cultured with conditioned media from co-cultured blastocyst, scanning electron micrographs demonstrated that antibodies had clearly interrupted cell-to-plastic adhesion (Figure 5C), but not cell-to-cell adhesion (Figure 5F, 5I). The EEC appeared generally similar to those cultured without antibodies with the exception of the retraction fibres (Figure 5F, 5I).

Dose–response experiments further demonstrated an up-regulation of β3 positive cells when IL-1α + IL-1β were added to the medium at a concentration of 10 pg/ml compared with control medium without added cytokines (40 versus
Figure 3. Concentrations of interleukin-1 (IL-1)α, IL-1β (A), IL-1ra (B) and the ratio IL-1α + IL-1β/IL-1ra (C) in 24 h conditioned media from embryo–endometrial epithelial cells cultured with single human embryos which correspond to the IL-1 producer population. Embryonic developmental stage refers to the stage in which the embryo had reached on the day that conditioned media was removed. *The IL-1α + IL-1β/IL-1ra ratio in blastocysts is significantly lower compared with 4- and 6-cell stages (P < 0.05). (Reproduced with permission of Biology of Reproduction from De los Santos et al., 1996).
Figure 4. Effect of the neutralization of the interleukin-1 (IL-1) system on blastocyst up-regulation of endometrial \( \beta_3 \) integrin. Results are expressed as the percentage of \( \beta_3 \)-stained cells of EEC monolayers cultured for 24 h in the presence of (i) a pool of conditioned media from co-cultured blastocyst (b.c.m.), (ii) a pool of conditioned media from co-cultured blastocyst with anti-human IL-1\( \beta \) antibody (5 \( \mu \)g/500 \( \mu \)l) + anti-human IL-1\( \alpha \) antibody (500 \( \mu \)g/500 \( \mu \)l), a pool of conditioned media from co-culture blastocyst with anti-human IL-1ra antibody (1\( \mu \)g/500\( \mu \)l), a pool of conditioned media from co-cultured blastocyst with recombinant human IL-1ra (10 \( \mu \)g/500 \( \mu \)l), or a pool of conditioned media from co-cultured blastocyst with anti-human leukaemia inhibitory factor (LIF) (250 \( \mu \)g/500 \( \mu \)l). Data are expressed as mean ± SEM and analysed by analysis of variance (ANOVA) followed by Scheffé’s F test. *Significance between groups (\( P < 0.05 \)). (Reproduced with permission from the Journal of Clinical Endocrinology and Metabolism from Simón C et al., 1997a).

The functional relevance of the EEC \( \beta_3 \) up-regulation was tested using a mouse blastocyst adhesion assay. More mouse blastocysts attached to EEC previously in contact with human blastocyst (72.7%) compared with those EEC previously in contact with arrested embryos (40%) (Simón et al., 1998).

**Discussion**

The results reported herein demonstrate, for the first time at the molecular level, the existence of a specific cross-talk between the embryo and endometrium in the adhesion phase of human implantation. This pathway of molecular interactions seems to be initiated by the endometrium in the presence of an implanting blastocyst. It is mediated through embryonic IL-1\( \alpha \) + IL-1\( \beta \), and the target is the up-regulation of endometrial epithelial \( \beta_3 \) integrin. If we accept the relevance of \( \beta_3 \) as a marker of uterine receptivity (Tabibzadeh, 1992; Lessey et al., 1992, 1994), these observations may imply that the normal hormonally-regulated human endometrium is the trigger of molecular events preparing the blastocyst to efficiently communicate and regulate the endometrium in order to implant.

20%, \( n = 4 \)).
After adhesion, the next step in the implantation process is the penetration of the blastocyst deep into the endometrial stroma. An interesting model has been proposed in mouse by which trophoblast invasion is promoted by proteases such as urokinase-type plasminogen activator (u-PA) (Strikland et al., 1976). Once initiated, invasion must be tightly regulated. Decidual cells might limit invasion by loading latent transforming growth factor-β (Lala and Graham, 1990) in response to plasmin, and TGF-β could restrict invasion via PA inhibitor induction (Laiho and Keski-Oja, 1989). Proteases would be complexed with inhibitors thereby decreasing the invasive ability of the trophoblast. Low density lipoprotein (LDL) receptor-related protein (LRP) mediates the internalization of receptor–proteinase–inhibitor complexes, so allowing free protease recycling in order to became available again and continue invasion (Herz et al., 1992).

Based on our research line, we propose a model (Figure 2) to understand the molecular mechanisms underlying the adhesion of the blastocyst to the endometrial epithelium. In response to an unknown endometrial epithelial factor, the human
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blastocyst is able to secrete the complete IL-1 system (IL-1α, IL-1β /IL-1ra) which is constitutively present but not secreted, as demonstrated by the selective release in the presence of EEC (De los Santos et al., 1996). The binding and activation of embryonic IL-1α + IL-1β to the endometrial epithelium induces an up-regulation of β3 subunit (Simón et al., 1998). Endometrial β3 up-regulation is morphologically important and functionally relevant because it increases the ability of the blastocyst to adhere to the EEC monolayer (Simón et al., 1998). In summary, this model provides evidence that the endometrium triggers a mechanism whereby the human blastocyst modulates human endometrial receptivity.

References


Masuhiro, K., Matsuzaki, N., Nishino, E. et al. (1991) Trophoblast-derived interleukin-1 (IL-1)
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