Novel insights into human endometrial paracrinology and embryo–maternal communication by intrauterine microdialysis

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The regulation of human implantation is still unknown. Evidence from mice suggests an essential role for several paracrine mediators but species differences with implantation in the human preclude the extrapolation of these concepts to humans. An intrauterine microdialysis device (IUMD), consisting of microdialysis tubing glued into a balloon catheter on one side and into a polypropylene tube on the other, allows a dynamic and accurate in-vivo measurement of uterine paracrine interactions in humans. Inserted into the uterine cavity in the form of a loop, it can be continuously perfused with saline to reveal a number of relevant cytokines and growth factors in uterine effluents of non-pregnant women in both follicular and luteal phases. These included interleukin (IL)-1α, IL-1β, IL-6, leukaemia inhibitory factor (LIF), macrophage colony-stimulating factor (M-CSF), epidermal growth factor, vascular endothelial growth factor (VEGF), insulin-like growth factor binding protein-1 (IGFBP-1), prolactin, and human chorionic gonadotrophin (HCG). The source of intrauterine HCG is unclear since endometrial mRNA for the HCG β-subunit is not revealed using reverse transcriptase polymerase chain reaction analysis. Applying urinary HCG locally via the IUMD profoundly alters endometrial secretory parameters. Prolactin, IGFBP-1, and M-CSF are significantly inhibited and VEGF is regulated in a biphasic manner involving early stimulation followed by inhibition of intrauterine levels. Use of the IUMD has thus shown that the urinary HCG preparations routinely used for ovulation induction and luteal support may directly alter endometrial function.

Key words: HCG/ implantation/microdialysis/uterine receptivity/VEGF

Introduction

The improvement of implantation rates remains one of the big challenges in human assisted reproduction. During the last few years, considerable progress has been made towards a better understanding of the processes underlying implantation in laboratory rodents. Based on these data it is clear that the preparation of the endometrium for implantation is probably achieved by the coordinate actions of different mediators. First, there is little doubt that the secretory transformation of the endometrium by steroids is an essential prerequisite. In addition, however, we have learned that a paracrine ‘fine tuning’ that is mediated by cytokines and growth factors is also necessary for an appropriate decidualization. This fine tuning seems to depend on a coordinated dialogue between the endometrium and the incoming blastocyst, which is referred to as embryo–maternal cross-talk.

A regulatory network of cytokines and growth factors has been demonstrated in the endometrium (Tabibzadeh, 1991) and there is evidence that at least some of these mediators are essential for the implantation process (Giudice, 1994; Harvey et al., 1995; Tabibzadeh and Babaknia, 1995). Elegant studies using ‘knock-out’ mice have shown that the functional deletion of the genes for
leukaemia inhibitory factor (LIF) (Stewart et al., 1992), macrophage colony-stimulating factor (M-CSF) (Pollard et al., 1991) and epidermal growth factor (EGF) (Harvey et al., 1995) disrupts implantation, while follicular maturation, ovulation and impregnation remain unaffected. Moreover, the interleukin 1 (IL-1)/interleukin 6 (IL-6) system seems to be important for the implantation process (Simon et al., 1994a,b).

Being one of the earliest embryonic signals, human chorionic gonadotrophin (HCG) may be a major mediator of the embryo–maternal communication. Specific receptor sites for HCG have been demonstrated in the endometrium with peak values in the luteal phase (Reshef et al., 1990), and there is evidence in vitro that HCG may be involved in the regulation of decidualization (Han et al., 1996) and implantation (Yagel et al., 1993). The majority of the studies have been performed either in the mouse system or in vitro with human tissue. However, because of the differences in the implantation process between the two species, these experiments cannot simply be extrapolated to the human system. Thus investigations on human tissue both in vitro and in vivo are essential for a better understanding of the complex paracrine interactions required for a successful implantation. As an approach, we have recently developed an intrauterine microdialysis device. We here present initial data on the clinical evaluation of the system.

**Intrauterine microdialysis**

Plasma-filter dialysis units PF 2000 (molecular weight cut-off: 2000 kDa) were purchased from Gambro Dialysatoren GmbH (Hechingen, Germany). Silastic balloon catheters (8 charrière) were from Uromed (Oststeinbeck, Germany). The components of the system were glued using silicone elastomer medical adhesive MED 1511 (NuSil Silicone Technology, Carpinteria, CA, USA). A multichannel high-precision peristaltic pump taken from a Biostator glucose monitor LS 3001 (Miles Laboratories Inc., Elkhart, USA) was used for perfusion. Recombinant cytokines (IL-1α, IL-1β, IL-6, M-CSF, EGF) were purchased from DPC (Minneapolis, MN, USA) as distributed by Biermann GmbH, Bad Nauheim, Germany. Immunoassays for LIF, EGF, M-CSF, IL-1α, IL-1β, IL-6, and vascular endothelial growth factor (VEGF) were from DPC, while an enzyme-linked immunosorbent assay for insulin-like growth factor binding protein-1 (IGFBP-1) was purchased from Diagnostic Systems Laboratories (Sinsheim, Germany). Intact HCG was determined using a chemoluminescence assay (Stratus system, Baxter, Unterschleissheim, Germany). Serum luteinizing hormone (LH), prolactin, progesterone and oestradiol were measured by enzyme immunoassays (ES 700, Boehringer Mannheim, Mannheim, Germany).

The construction of the intrauterine microdialysis device (IUMD) is discussed in detail elsewhere (A.Lösch et al., manuscript in preparation). Briefly, the IUMD consists of a 8-charrière balloon catheter connected to a 16 G polypropylene catheter through plasma-filter membranes (molecular weight cut-off: 2000 kDa). It is inserted into the uterine cavity in the form of a loop and gently blocked (Figure 1). Perfusion with sterile saline is performed over a period of up to 7 h by simultaneous pumping and suction, using a precision peristaltic pump (Biostator, Miles Lab. Inc., Elkhart, USA) at a flow rate of 30 µl/min. The effluent is collected in fractions of 1 h (1.8 ml) and frozen immediately at −80°C. Gradients were established by pumping sterile saline through microdialysis tubing that allows the penetration of molecules up to a molecular weight of 2000 kDa by diffusion. Since time does not allow for a total equilibration of concentrations, a steady state was reached that was shown to be dependent on several factors, such as molecular weight, flow rate, temperature and surface of the membranes (A.Lösch et al., manuscript in preparation). The determination of the recovery for each individual system and mediator by means of in-vitro microdialysis of recombinant cytokine cocktails allows the calculation of secretion rates when the steady-state is reached. In addition, it is possible to obtain information on the secretion dynamics of a given mediator and to assess the response to treatment as discussed below.
Dynamic microdialysis over short periods of time does not allow for a total equilibration of concentrations between uterine fluid and the perfusate. Thus, the individual recovery factor for each catheter had to be determined in vitro in order to allow for a calculation of actual concentrations within the uterine cavity. This was achieved by in-vitro microdialysis of a cytokine cocktail. Recombinant cytokines and growth factors (EGF, 3000 pg/ml; LIF, 30 000 pg/ml; IL-1α, 3000 pg/ml; IL-1β, 3000 pg/ml; IL-6, 1.428 pg/ml; M-CSF, 30 000 pg/ml) were dissolved in saline and microdialysis was carried out in vitro using the same catheter after the IUMD had been removed from the uterus. The recovery for HCG was determined by in-vitro microdialysis of early pregnancy serum. Temperature was adjusted to 37°C by the use of a water bath. The individual recovery factor was calculated as concentration of the uterine effluent divided by concentration of the original sample. Mean recovery factors (± SD) were: LIF (5.6% ± 3.0), M-CSF (7.3% ± 3.4), IL-1α (11.5% ± 5.8), IL-1β (11.6 ± 6.2), IL-6 (5.2% ± 1.3), EGF (14.1% ± 10.4), and HCG (8% ± 3.0).

Clinical and experimental studies

For the initial clinical evaluation of the IUMD, six normal cyclic women were recruited from our infertility set-up. All of them suffered from unexplained infertility. There were no significant endocrine abnormalities. Informed consent was obtained in writing before a diagnostic cycle could be initiated. Starting on day 10, blood samples were collected daily and assayed for oestradiol and LH. The size of the dominant follicle was measured by vaginal ultrasonography. Thereafter, daily blood collection was continued until the preovulatory LH-peak occurred. The size of the follicular phase when oestradiol levels were above 100 pg/ml and a single dominant follicle was seen by vaginal ultrasonography. Thereafter, daily blood collection was continued until the preovulatory LH-peak occurred. The appointment for the second intrauterine microanaylsis procedure was performed in the late follicular phase when oestradiol levels were above 100 pg/ml and a single dominant follicle was seen by vaginal ultrasonography. The time period of the LH-peak occurred. The appointment for the second intrauterine microanaylsis procedure was set on days 7–9 after the start of the LH-peak, and the third examination was held on days 10–12 during the late luteal phase.

The HCG test was carried out on a total of eight normal cyclic women in the late luteal phase (LH-peak + 10–12 days). The study protocol was explained and informed consent was obtained in writing before a cycle monitoring was initiated as explained above. Following equilibration of the system in utero for 1 h, the system was perfused with sterile saline only for another 2 h in order to obtain basal values. Thereafter HCG infusion was started at a concentration of 500 IU urinary HCG (Choragon®, Ferring, Germany) per hour and was continued for 5 h. Assuming a penetration rate of about 10% for HCG through the microdialysis tubing, which had been determined by in-vitro microdialysis, the actual concentrations applied to the endometrium were estimated to be around 50 IU/h. To control for endocrine effects of the HCG applied, blood samples were withdrawn at hourly intervals before and during HCG infusion and assayed for HCG and progesterone.

Storage of the samples and cytokine analysis

Immediately after being collected, the samples were frozen and stored at –80°C in sterile tubes. The samples were thawed shortly before the immunoassays were carried out, avoiding repeated freezing and thawing. Immunoconcentrations for LIF, EGF, M-CSF, IL-1α, IL-1β, IL-6, VEGF, IGFBP-1, prolactin and HCG were determined as duplicates using commercial immunoassays according to the protocol provided by the manufacturer. There were no significant cross-reactivities among the cytokines tested. The intra- and inter-assay coefficients of variation were below 5%.

Isolation of RNA and reverse transcriptase polymerase chain reaction (rT-PCR) determination of mRNA for the β-subunit of HCG

Endometrium tissue was obtained from premenopausal women undergoing hysterectomy because of leiomyomata uteri. The tissue was snap-frozen in liquid nitrogen and total cellular RNA was extracted by phenol/chloroform extraction (RNA clean®, AGS, Heidelberg, Germany). Five micrograms of total cellular RNA were subsequently reverse transcribed for 60 min at 42°C using the down-stream primer for HCG-β and random hexamers for β-actin. Subsequently, amplification with primers specific for the β-subunit of HCG (covering the genes 3, 5, 7 and 8) was carried out as previously reported for 30 cycles [1′ at 95°C, 2′ at 60°C, 3′ at 75°C] (Bo and Boime, 1992). The sense primer used was 5′-TCGGGTCAAGGCCTCTCCT-3′, the antisense primer was 5′-CCGGGACACCCCTGCAGCA-3′. Reaction conditions were 0.025 U/µl Taq polymerase (Perkin–Elmer, Überlingen, Germany), 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM MgCl2, 200 µM dNTPs, 1.0 µM Primers. The same samples were also analysed for the expression of β-actin mRNA under identical conditions. This ensured the presence of intact RNA in all samples. Primers for β-actin were 5′-CCAGGCCAGGGGCA-TAT-3′ and 5′-TCAAACTGATCTGTCAT-3′. The PCR products were subsequently analysed by
Intrauterine microdialysis

Figure 2. Intrauterine concentrations of cytokines as measured using the intrauterine microdialysis device. A typical example for one woman is depicted. Microdialysis was performed three consecutive times within one menstrual cycle. F = late follicular phase; L1 = mid luteal phase; L2 = late luteal phase; IL-1 = interleukin 1; IL-6 = interleukin 6; LIF = leukaemia inhibitory factor; EGF = epidermal growth factor; M-CSF = macrophage colony-stimulating factor.

Figure 3. Secretion dynamics of the proinflammatory cytokines interleukin (IL)-1β and IL-6. Following equilibration of the system in utero, three fractions of 45 min were collected and assayed for IL-1β and IL-6. Both cytokines consistently increased following insertion of the intrauterine microdialysis device. The slope of the rise was most pronounced in the luteal phase of the menstrual cycle.

electrophoresis in a 2% agarose gel and staining with ethidium bromide. Molecular weight was estimated by running molecular weight markers in an adjacent lane.

Paracrine and endocrine mediators in uterine secretions

Using the IUMD, we were able to confirm the presence of a variety of cytokines and growth factors, which are considered important in the rodent, in the uterine fluid of the human female. LIF, M-CSF, IL-1β and IL-6 tended to be higher in the luteal phase than in the follicular phase (Figure 2). The secretion kinetics of the proinflammatory cytokines IL-1β and IL-6 were unique. In contrast to all the other mediators that reached a plateau, IL-1β and IL-6 consistently increased following insertion of the system. The slope of this rise seemed to be more pronounced in the late luteal phase of the menstrual cycle (Figure 3).

The uterine perfusate of several women contained immunoreactive (ir-) HCG (Figure 4A). In order to address the source of this ir-HCG, an rT-PCR analysis for the expression of mRNA for the β-subunit of HCG was performed. While placenta tissue expressed the predicted 562 bp cDNA, human endometrium obtained from premenopausal patients was negative for HCG-β mRNA (Figure 4B). Simultaneous amplifications with β-actin ensured the presence of intact RNA in all samples.

The application of low concentrations of urinary HCG into the uterine cavity profoundly altered endometrial secretory parameters. Prolactin, IGFBP-1 and M-CSF declined significantly, starting ∼1 h following HCG infusion and reaching a maximum after 5 h at the end of infusion (Figure 5A). LIF secretion was consistently increased by 200–300% as compared with control values. Intrauterine VEGF levels followed a biphasic regulation with an initial increase followed by a decrease (Figure 5A). During the entire procedure, no ir-HCG could be measured in the peripheral circulation (HCG <5 mIU/ml). In addition, the HCG infusion did not alter progesterone secretion by the corpus luteum (Figure 5B).

During the last 30 years, several efforts have been undertaken to determine in-situ concentrations of paracrine mediators. The push–pull technique invented by Gaddum (Szerb, 1967) allowed in-vivo measurements of neurotransmitters for the first time. This method, however, had shortcomings, e.g. the injury of the perfused tissue by the compression of the pushing liquid and the resulting contamination of the samples with blood and tissue pieces. Essentially the same is true for uterine flushings that have been applied in some recent studies addressing the presence of cytokines in the uterine fluid (Todorow et al., 1993; Mercander et al., 1996). The microdialysis technique represents a less invasive sampling method since
there is no direct contact between liquid and tissue (Kendrick, 1989). The approach presented here is a modification of this technique that allows an accurate and dynamic in-vivo measurement of uterine paracrine mediators in humans.

Using the IUIMD, we were able to confirm the presence of most of the mediators that are considered essential for implantation in the mouse also within the uterine fluid of the human female, in the follicular as well as in the early and late luteal phase of the menstrual cycle. Even though the low number of patients did not allow meaningful statistical analyses to be performed, LIF, M-CSF, IL-1β and IL-6 tended to be higher in the luteal phase than in the follicular phase. These findings confirm the observations of Ämmälä et al. (1995), who reported higher mRNA levels for IL-1β, IL-6 and TNF-α in the secretory phase than in the follicular phase.

Several lines of evidence point towards a parallelism between the processes underlying blastocyst implantation and an inflammatory reaction. This view is strengthened by the results of the present study. While all other mediators studied reached a plateau after a maximum of 2 h of microdialysis, the immunoreactive levels of the
proinflammatory cytokines IL-1β and IL-6 consistently increased following insertion of the IUMD. The slope of this rise seemed to reach its maximum in the late luteal phase, suggesting a more pronounced inflammatory reaction in this phase of the menstrual cycle. An overshooting inflammation may explain some abortions as well as the therapeutic effect of steroids in the treatment of a subgroup of patients with habitual abortions. Experiments are currently under way to determine a possible direct effect of prophylactic steroids on endometrial secretion in vivo.

It is a noteworthy feature that implantation of the blastocyst can take place virtually everywhere in the human body except for the non-receptive endometrium. Endometrial receptivity, however, is tightly restricted to a narrow time frame following ovulation. While we know some factors that define the implantation window, such as the integrin pattern (Lessey et al., 1995), we know very little about the regulation of uterine receptivity. Being one of the earliest embryonic products, HCG may be an important mediator in this regard. HCG is normally secreted by the syncytiotrophoblasts of human placenta. Recently, our laboratory (Dittrich et al., 1997) as well as others (Alexander et al., 1997) have found immunoreactive HCG in uterine fluid and endometrium of non-pregnant women. Using the IUMD, we were able to detect ir-HCG in the effluent of some, but not all, women under investigation. The source of HCG within the uterine fluid is not clear at present. However, in contrast to placenta, human endometrium obtained from premenopausal women undergoing hysterectomy because of leiomyomata uteri did not express messenger RNA for the β-subunit of the hormone.

HCG has been implicated in several tissue functions throughout the female reproductive system. Besides its well-known effects on steroidogenesis, there seem to be a variety of functions covering the entire male and female reproductive system. In the placenta, HCG is able to self-regulate its own biosynthesis via specific receptors (Licht et al., 1993) and to stimulate trophoblast differentiation (Shi et al., 1993). These functions, however, seem to be restricted to the later stages of pregnancy since early trophoblasts do not express full-length HCG/LH receptors (Licht et al., 1994a,b). Furthermore, HCG/LH-receptors have been demonstrated in human endometrium with peak values in the luteal phase of the menstrual cycle (Reshef et al., 1990; Lin et al. 1994), and there is evidence that HCG may be involved in implantation by modulating the activity of collagenases and plasminogen activators in an in-vitro system (Yagel et al., 1993) and directly promoting decidualization (Han et al., 1996). Moreover, it is a noteworthy feature, that most of the mediators considered essential for the implantation process (LIF, M-CSF, EGF, IL-1/IL-6) have previously been shown to be also involved in the regulation of HCG biosynthesis by the placental syncytiotrophoblasts (Beneviste et al., 1978; Masuhiro et al.; 1991; Harty and Kauma, 1992; Sawai et al., 1995), probably by modulating trophoblast differentiation. Therefore, we postulated that HCG may play a central part in the hypothetical embryo–maternal cross-talk.

To test this hypothesis, we have simulated the effect of a very early pregnancy on the decidualized endometrium by the application of very small doses of urinary HCG (500 IU/h) into the uterine cavity of eight normal cycling women in the late luteal phase (HCG test). Under these conditions, no immunoreactive HCG was found in the peripheral circulation (HCG <5 mIU/ml) and the treatment did not alter progesterone secretion by the corpus luteum, suggesting that the effects observed were direct. Urinary HCG consistently and significantly decreased a variety of parameters considered to correlate with the degree of decidualization (prolactin, IGFBP-1, M-CSF). On the other hand, LIF, a cytokine considered essential for implantation in the rodent, was consistently increased by HCG, and VEGF, a potent endothelial mitogen and vascular permeability factor that is thought to be important for placenta, was regulated in a biphasic manner with an early increase followed by a decrease of immunoreactive levels.

In this study we have used a urinary preparation of HCG that is routinely used for ovulation induction and luteal support in assisted reproduction. We are well aware that these preparations contain significant contaminations with other peptides and proteins secreted during early pregnancy. In particular, EGF is present in high concentrations in all commercial preparations (P.Licht, O.Fischer, L.Wiett, et al., manuscript in preparation). To find out whether the effects observed are due to HCG or to a contamination, in-vitro experiments will be conducted to compare the effects of urinary HCG and recombinant HCG on endometrial cytokine production.

Taken together these initial data show that the putative key players in the regulation of implantation are secrected into the uterine cavity in measurable amounts. We suggest that IUMD is a non-invasive tool to measure these interactions. The IUMD was well tolerated by the patients. The insertion of the soft balloon catheter did not cause any pain or severe discomfort. This view is strengthened by the fact that all patients attended all three consecutive examinations within one menstrual cycle. There was no withdrawal. Although the patients were advised and
screened for inflammatory complications, we did not observe any inflammation or severe bleeding due to the intrauterine microanalysis procedure. The intrauterine microdialysis did not interfere negatively with the chances of the women becoming pregnant. Six out of 14 women with longstanding infertility conceived within two cycles following microdialysis.

In summary, the IUMD allows an accurate in-vivo estimation of intrauterine paracrine mediators in humans. Moreover, it allows dynamic control of the endometrial secretory response to exogenous stimuli and may be a useful tool to find out whether drugs used, e.g. for ovarian stimulation, have a direct effect on the endometrium. So far, HCG has never been shown to have a direct influence on endometrial functions in vivo. Using the IUMD, we demonstrated for the first time that HCG directly regulates the secretion of endometrial cytokines and growth factors, and thus may play an essential part during the initiation of pregnancy.

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References