Analysis of Fertility-Related Soluble Mediators in Human Uterine Fluid Identifies VEGF as a Key Regulator of Embryo Implantation

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Embryo implantation requires synchronized dialogue between the receptive endometrium and activated blastocyst via locally produced soluble mediators. During the midsecretory (MS) phase of the menstrual cycle, increased glandular secretion into the uterine lumen contains important mediators that modulate the endometrium and support the conceptus during implantation. This study aimed first to identify the growth factor and cytokine profile of human uterine fluid from fertile women during the midproliferative (MP; nonreceptive) and MS (receptive) phases of the cycle, and from women with unexplained infertility during the MS phase. The second aim was to determine important functions of endometrial secretions for embryo implantation. Analysis of uterine fluid using quantitative Luminex assays revealed the presence of over 30 cytokines and growth factors, of which eight [platelet-derived growth factor-AA, TNF-B, soluble IL-2 receptor-A, Fms-like tyrosine kinase 3 ligand, soluble CD40 ligand, IL-7, interferon-A2, and chemokine (C-X-C motif) ligand 1–3] were previously unknown in human uterine fluid. Comparison of the fertile MP, MS, and infertile MS cohorts revealed vascular endothelial growth factor (VEGF) levels are significantly reduced in uterine fluid during the MS phase in women with unexplained infertility compared with fertile women. Functional studies demonstrated that culturing mouse embryos with either MS-phase uterine fluid from fertile women or recombinant human VEGF significantly enhanced blastocyst outgrowth. Furthermore, treatment of human endometrial epithelial cells with uterine fluid or recombinant human VEGF-A significantly increased endometrial epithelial cell adhesion. Taken together, our data support the concept that endometrial secretions, including VEGF, play important roles during implantation. Identifying the soluble mediators in human uterine fluid and their actions during implantation provides insight into interactions essential for establishing pregnancy, fertility markers, and infertility treatment options. (*Endocrinology* 152: 4948–4956, 2011)

The human endometrium is receptive to blastocyst implantation for only approximately 4 d in the midsecretory (MS) phase of each menstrual cycle (1). Deficiencies in endometrial receptivity can influence infertility through implantation failure and early pregnancy loss and impact the outcome of *in vitro* fertilization (IVF).

During the secretory phase and controlled by rising progesterone levels, endometrial glandular epithelial cells transform from relatively inactive to highly secretory polarized cells that transport or synthesize and secrete substances into the uterine lumen. Aside from being a hallmark of the secretory phase, these secretions are likely to

Abbreviations: CCL2, Chemokine (C-C motif) ligand 2; CXC10, chemokine (C-X-C motif) ligand 10; ECM, extracellular matrix; FLT3L, Fms-like tyrosine kinase 3 ligand; hCG, human chorionic gonadotropin; HSA, human serum albumin; IVF, *in vitro* fertilization; MP, midproliferative; MS, midsecretory; PDGF-AA, platelet-derived growth factor-AA; rh, recombinant human; sCD40L, soluble CD40 ligand; sIL2RA, soluble IL-2 receptor-A; VEGF, vascular endothelial growth factor; VEGFR1, VEGF receptor 1.

be important mediators of blastocyst development and endometrial function. A number of studies have examined the nature of endometrial secretions in certain animal species and throughout the menstrual cycle in humans (2–5). These studies have often been biased toward particular molecules of interest or have revealed little information on the specific proteins secreted into the lumen (6). Binding and nutrient transport proteins, ions, glucose, cytokines, enzymes, hormones, growth factors, proteases and their inhibitors, and other substances are produced and secreted by the glands (7–9). Such material is thought to provide important nutritional factors during the early stages of implantation and placentation. In addition, these secretions may play broader roles in regulating endometrial receptivity, immunomodulation, and placental morphogenesis. The importance of histotroph from uterine glands is emphasized in a sheep model where uterine gland formation is inhibited (10) and pregnancy cannot be established. A variety of growth factors and cytokines have been identified in uterine glands in women, although it remains unknown whether they are secreted and retain their bioactivity within the uterine lumen (11). Circumstantial evidence suggests that deficient glandular activity, usually described as a secretory-phase defect, could result in early pregnancy failure (12, 13). However, there is no conclusive evidence to support this in the human. Biochemical assessments of glandular activity have shown that the concentration of glycodelin A in uterine flushings on menstrual cycle d 24–26 are lower in women who subsequently miscarry than in those with successful pregnancies (14). Furthermore, disrupted secretion of individual soluble factors including cytokines, growth factors, and proteases into the uterine lumen has been correlated with infertility (5, 13, 15). Whether infertility or early miscarriage results from lack of adequate embryo nutrition, defective placentation, or abnormal immunological interactions at the maternal fetal interface is not yet known. Studies identifying the products of glandular secretions, bioactivity, and function are therefore a necessary prerequisite to further understanding. Furthermore, there are currently no definitive markers of endometrial receptivity that can reliably identify receptive endometrium during IVF procedures.

Previously, we and others have examined changes in the human endometrial secretome using a proteomics approach, revealing novel differentially regulated proteins not previously known to be secreted by the human endometrium as well as important networks among the proteins that are enriched in the receptive endometrium (16, 17). Furthermore, analysis of a cohort of selected cytokines and chemokines in aspirates of uterine fluid collected from women undergoing IVF or intracytoplasmic sperm

injection were assessed by a multiplex immunoassay. These studies demonstrated the presence of 16 soluble mediators in the aspirates, with considerable variability between women (15, 18). Despite the patient variability, significant associations were observed between chemokine (C-C motif) ligand 2 [CCL2 (monocyte chemoattractant protein-1)] and chemokine (C-X-C motif) ligand 10 [CXCL10 (IP-10)] levels in subjects with successful implantation and between IL-1B and TNF-A levels in those with clinical pregnancy (18).

The present study expanded our knowledge of endometrial secretory changes associated with receptivity by applying multiplex assays to determine changes in 42 cytokines, chemokines, and growth factors in endometrial lavage, with cycle stage (nonreceptive *vs.* receptive) and with fertility status. Some of the factors identified were not previously known in the endometrium. Importantly, we have demonstrated that one of the key regulated factors, vascular endothelial growth factor (VEGF), has substantial effects on both endometrial epithelial adhesive capacity and on blastocyst outgrowth.

Materials and Methods

Ethics

Ethical approval was obtained from Monash Surgical Private Hospital and Human Research Ethics Committee (Project No. 04056) and Southern Health Human Research Ethics Committee (Project No. 03066B) for all human sample collections. Written informed consent was obtained from all subjects before sample collection. All animal experimentation was conducted in accord with accepted standards of humane animal care, as outlined in the Ethical Guidelines. Ethical approval was obtained from The University of Melbourne Animal Ethics Committee (Project ID 0811074.2) before experimentation.

Sample collection and patient details

Human endometrial lavages and biopsies [a total of n = 7–9 patients per group (phase of the cycle); Table 1] were obtained during the nonreceptive, midproliferative (MP; d 8–11) and receptive, MS (d 19–23) phases of the menstrual cycle, from women with proven fertility or from the MS phase of women

TABLE 1. Summary of patient details

Endometrial tissue and lavage samples	Age (yr)	Histological day of cycle	Number per group
Fertile			
MP	34.7 ± 2.3	8–11	7
MS	34.4 ± 2.9	19–23	8
Infertile			
MS	33.6 ± 4.4	19–23	9

Samples used, number of samples per group, histological dating of menstrual cycle phase, and fertility status are indicated.

with unexplained infertility who were undergoing hysteroscopy, dilatation, and curettage. The infertile women had been screened for nonendometrial causes of their infertility and tubal patency, and their partner did not have male factor infertility. Patients with uterine abnormalities such as endometrial polyps, endometriosis, or endometritis or who had received steroid hormone therapy in the last 6 months were excluded. In brief, 5 ml sterile saline was infused into the uterine cavity through a fine flexible catheter; the saline solution was then aspirated, centrifuged to remove cellular debris, and stored at -80 C as 0.5-ml aliquots. Samples of endometrial curettings (performed after lavage) were formalin fixed overnight (16 $\pm$ 1 h) at 4 C, washed three times in Tris-buffered saline (pH 7.6), and stored at 4 C until wax embedding. Menstrual cycle stage was confirmed by histological dating of tissue (19).

Lavage concentration

One milliliter of each lavage sample was loaded onto Microsep UF spin filter columns (3 kDa molecular mass cutoff) (PALL Life Sciences, Port Washington, NY) centrifuged at $4500 \times g$ and the volume reduced 4-fold. The retentates were transferred to fresh tubes and either assessed using Luminex assays or filtered through 0.22-μm filters for blastocyst outgrowth experiments and endometrial epithelial adhesion assays.

Luminex multiplex assays

Initially, 42 cytokines, chemokines, and growth factors were assayed simultaneously in uterine fluid (n = 4 women per group) using quantitative Milliplex Luminex (MilliPlex MAP Human Cytokine Panel 42plex; Millipore, Melbourne, Victoria, Australia) assays according to the manufacturer's instructions. In brief, 96-well plates were prewetted with 200 μl assay buffer (provided by manufacturer) for 10 min and then aspirated using a vacuum manifold. Standards and samples (25 μl) were added to appropriate wells, followed by addition of assay beads. Plates were incubated overnight (16–18 h) with mild agitation at 4 C, the fluid removed by vacuum, and the wells washed twice with wash buffer. Detection antibodies were added to each well and incubated for 1 h at room temperature, the fluorescent conjugate streptavidin-phycoerythrin added to each well, and plates incubated for 30 min at room temperature. Fluid was then removed by vacuum and wells washed twice. Analysis of each sample was performed in duplicate. To avoid interassay variation, all samples compared statistically were analyzed on the same plate. The lower detection limit was 4 pg/ml for all analytes, and intraassay variability was less than 10%. Data were collected and analyzed using a BioPlex 200 instrument equipped with BioManager analysis software (Bio-Rad, Hercules, CA). Test runs were performed before analyses of the full set of samples to optimize the sample dilution such that each analyte was analyzed within the functional range of its standard curve.

VEGF levels in additional uterine fluid samples were assessed in an independent Milliplex Luminex assay. The assay was carried out as described above, examining uterine fluid collected from fertile women during the MP (n = 7) and MS (n = 8) phase and from women with unexplained infertility during the MS (n = 9) phase; these included the n = 4 per group previously analyzed.

Animals and hormonal stimulation

Pronucleate oocytes were obtained from F1 hybrid (C57BL/6 $\times$ CBA/Ca) mice. Six-week-old females were superovulated with ip injections of 5 IU pregnant mare's serum gonadotropin (Folligon; Intervet, Cambridge, UK) followed 48 h later by 5 IU human chorionic gonadotropin (hCG, Chorulon; Intervet). Females were mated with F1 males overnight after hCG injection. The presence of a vaginal plug the following morning was used as an indicator of successful mating.

Embryo collection and culture

Pronucleate oocytes were collected approximately 22–24 h after hCG in G-MOPS medium (20) supplemented with 5 mg/ml human serum albumin (HSA) (Vitrolife, Gothenburg, Sweden), followed by cumulus removal in G-MOPS containing 300 IU/ml hyaluronidase (bovine testes, type IV; Sigma-Aldrich, St. Louis, MO). Pronucleate oocytes were removed from the hyaluronidase immediately once the cumulus cells had detached, washed twice in G-MOPS, and then once in G1 medium (21, 22) before culture. Embryos were cultured in 20-μl drops of G1 medium supplemented with 5 mg/ml HSA under paraffin oil (Ovoil; Vitrolife) at 37 C in 6% CO_2, 5% O_2, 89% N_2. After 48 h, all embryos were transferred into G2 medium (21, 22) supplemented with 5 mg/ml HSA under the same gas-phase conditions for an additional 30 h.

Experimental embryo culture

After 78 h of culture, embryos were transferred to 20-μl drops of one of six experimental treatments as follows: a control group (G2 medium with 5 mg/ml HSA); VEGF test group dose response containing recombinant human (rh)VEGF (R&D Systems, Minneapolis, MN) at doses of 5, 50, and 500 ng/ml in G2 control medium; and two test groups containing human uterine lavage (50 ng/ml total protein, pooled and concentrated as above) collected from women in the proliferative phase or the MS phase in control medium. Embryos were cultured in their respective experimental medium for 24 h to the hatched blastocyst stage (approximately 126 h after hCG) under paraffin oil (Ovoil) at 37 C under the same gas-phase conditions.

Embryo outgrowths

Flat-bottomed 96-well tissue culture dishes (BD Biosciences, San Jose, CA) were rinsed twice with sterile PBS and coated with a solution of fibronectin (10 μg/ml). The coated wells were rinsed twice with sterile PBS and incubated with a 4 mg/ml BSA (Sigma Diagnostics, St. Louis, MO) PBS solution. Wells were rinsed with PBS and then G2 medium and subsequently filled with 150 μl of appropriate experimental medium and equilibrated at 37 C under paraffin oil (Ovoil) under the same gas-phase conditions for 3 h before the addition of blastocysts. Hatched blastocysts that had been precultured in appropriate medium were placed individually into the coated wells (one embryo per well) and incubated for 96 h. Outgrowth was examined and images were taken at a matching magnification ($\times$10) at various times (66, 74, 90, and 98 h after transfer to outgrowth plate) during the culture period with an inverted microscope (Eclipse TS100-F; Nikon, Coherent Scientific Pty. Ltd., South Australia, Australia) equipped with a heated stage at 37 C. At the end of the experiment, embryo attachment in the absence of spreading was assessed by gently applying pressure to the blastocyst using a

pipette; blastocysts that were displaced were considered unattached. The extent of outgrowth for each treatment was obtained by measuring the area of outgrowth in each of the images taken across the experiment using NIS Elements BR version 3.00, SP7 Laboratory Imaging software (Nikon). All images were analyzed at matching magnification. The average area of outgrowth was calculated for each treatment with n = 15 embryos in total per treatment (n = 5 per experiment). The experiment was repeated three times. Data are expressed as mean outgrowth ± SEM.

Endometrial epithelial cell culture and adhesion assays

The human endometrial epithelial cell line ECC1 (23) was used in adhesion assays to determine the effects of both rh-VEGFA and uterine fluid on their adhesive capacity. The ECC1 cell line is considered the best available representative of luminal endometrial epithelial cells; what is known of their phenotype has been recently detailed (24). Alterations in ECC1 adhesive capacity was assessed with the CytoMatrix Screening Kit, ECM105 adhesion assays (Chemicon, Temecula, CA) as described previously (25). In brief, ECC1 cells were cultured in DMEM (Trace Biosciences) for tissue culture with 10% charcoal-stripped fetal calf serum for two to three passages after thawing. Once 80% confluent, cells were serum starved for 48 h in serum-free DMEM containing transferrin (10 μg/ml), sodium selenite (25 ng/ml), linoleic acid (4.7 μg/ml), and BSA (1 mg/ml) (all from Sigma Diagnostics, Castle Hill, NSW, Australia) (DMEM/TSL). Doses for ECC1 treatment were selected from the optimal doses observed in blastocyst outgrowth experiments. Cells were then cultured for an additional 24 h with 1) DMEM/TSL with 50 ng/ml BSA (control), 2) DMEM/TSL with 50 ng/ml rhVEGFA, or 3) DMEM/TSL with (50 ng/ml total protein) concentrated and sterile filtered uterine lavage fluid (pooled and concentrated as above) collected from women in the MS phase. Cells were then gently harvested and washed twice with sterile PBS, resuspended in DMEM/TSL, counted, and diluted to a final concentration of 10^6 cells/ml. Diluted cell suspension (100 μl) was added to each well of plates coated with either fibronectin or collagen IV matrix substrates. After incubation for 1.5 h at 37 C in a CO_2 incubator, the wells were gently washed three times with PBS containing Ca^{2+}/Mg^{2+}, 0.2% crystal violet in 10% ethanol (100 μl) was then added to each well, and the plate was incubated for 5 min at room temperature. Excess stain was gently removed, and wells were washed three times with PBS. The cell-bound stain was solubilized for visualization by the addition of 100 μl solubilization buffer [a 50/50 mix of 0.1 M NaH_2PO_4 (pH 4.5) and 50% ethanol] to each well and incubated at room temperature for 5 min. Adhesion was determined based on the absorbance of the stain measured at 540–570 nm. Negative control (BSA-coated) wells were included for each treatment in the assay. The entire experiment was carried out three times using identical conditions and treatments (with n = 5 wells per treatment in each experiment). Results are expressed as mean adhesion (expressed as fold change from control) ± SEM.

Statistical analysis

After testing for normal distribution, statistical analysis was performed on raw data. Luminex assays were assessed nonparametrically by Mann-Whitney U or by Kruskal-Wallis followed by Dunn's *post hoc* test. Both embryo outgrowth and endometrial epithelial cell adhesion assay data were normally distributed and were tested parametrically. Embryo outgrowth data were assessed using ANOVA followed by a Tukey-Kramer *post hoc* test. Adhesion assay data were analyzed by unpaired Student's t test. P values <0.05 were taken as significant. All statistical analysis was carried out using PRISM version 3.00 for Windows (GraphPad, San Diego, CA).

Results

Intrauterine cytokine and growth factor profile of human uterine fluid

Uterine lavage samples from each group (n = 4 per group; MP, MS fertile, and MS infertile) were initially assessed for their cytokine, chemokine, and growth factor profile (assessing 42 analytes in total). Table 2 shows the concentration ranges and the percentage of samples with detectable levels of the selected cohort of 42 cytokines and growth factors in human uterine lavage. Data are expressed as picograms per milliliter (original lavage volume, 5 ml) of unconcentrated fluid. For each analyte, the concentration range and percentage of women tested in whom levels were detected is shown. Thirty of the 42 analytes were detectable. Eight of these were not previously known in human uterine fluid; these were platelet-derived growth factor-AA (PDGF-AA), TNF-B, soluble IL-2 receptor-A (sIL2RA), Fms-like tyrosine kinase 3 ligand (FLT3L), soluble CD40 ligand (sCD40L), IL-7, interferon-A2, and CXCL1–3. Changes in analyte profile were assessed between fertile women from the nonreceptive MP and the receptive MS phase and between fertile women and those with unexplained infertility, during the MS phase. There was no significant difference in any of the analytes assessed; however, differences in VEGFA levels approached significance in uterine fluid from the MS phase when compared with the MP phase. Therefore, to examine whether VEGFA was altered across the cycle or with fertility, VEGFA levels were examined in larger patient groups with n = 7–9 (including the original samples) uterine lavage samples per group. With larger patient numbers, VEGFA levels were significantly reduced in uterine fluid collected from unexplained infertile women when compared with fertile women during the MS phase (Fig. 1). VEGFA levels were increased in the MS phase compared with the MP phase in fertile women, approaching statistical significance ($P = 0.0537$).

Effects of VEGFA and uterine fluid on blastocyst outgrowth

Mouse embryos were used to assess the functional effects of rhVEGF and uterine fluid on blastocyst out-

TABLE 2. Chemokines, cytokines, and growth factors present in human uterine lavage fluid

	% detectable	Range (pg/ml)
Chemokines		
CCL2	100	66–882
CCL3	17	5–36
CCL4	100	0.7–63
CCL5	100	6–1092
CCL7	100	0.3–29
CCL11	100	1.4–25
CCL22	100	1.13–486
CXCL1–3	100	31.3–5410
CXCL8	100	5–2503
CXCL10	100	44–2346
CX3CL1	100	4–71
Growth factors		
EGF	0	ND
FGF2	100	2.6–93
VEGFA	100	2–593
PDGFAA	100	15–651
PDGFAB/BB	42	3–195
TGFA	75	1.3–10
Receptors		
IL-1RA	100	1.2–387
sIL-2RA	75	0.8–18
sCD40L	92	1.46–96
Cytokines		
IL-1A	100	14–332
IL-1B	0	ND
IL-2	0	ND
IL-3	0	ND
IL-4	0	ND
IL-5	0	ND
IL-6	83	1.44–216
IL-7	100	0.88–15
IL-9	0	ND
IL-10	33	1.1–6.8
IL-12 (p40)	75	0.86–9.5
IL-12 (p70)	0	ND
IL-15	100	0.38–6.5
IL-13	0	ND
IL-17	0	ND
TNFA	0	ND
TNFB	8	0.46–0.55
IFNA2	100	1.0–7.9
IFNG	0	ND
GMCSF	83	1.09–22
GCSF	100	2.33–1116
FLT3L	75	1.5–7.5

The percentage of samples with detectable levels and the range (picograms per milliliter) are shown. Levels demonstrated are representative of n = 12 lavage samples for each factor except VEGFA, which is representative of n = 22 lavage samples. EGF, Epidermal growth factor; FGF, fibroblast growth factor; GCSF, granulocyte colony-stimulating factor; GMCSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; ND, nondetectable.
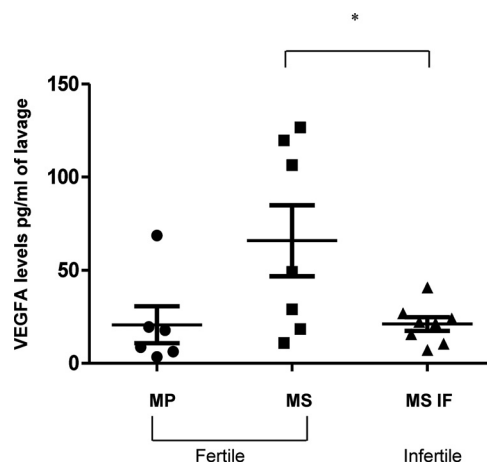
FIG. 1. Levels of VEGFA in human uterine lavage collected from fertile women during the MP (n = 7) and MS (n = 8) phase of the cycle and from infertile women during the MS phase (MS IF; n = 9). Results are expressed as a scatter plot demonstrating individual patient concentration of VEGF in picograms per milliliter of lavage, with the mean value represented by the *solid line*. *, $P < 0.05$.

$P < 0.01$ at 98 h after hatching). Doses of 5 and 500 ng/ml did not affect blastocyst outgrowth (Fig. 2A). Addition of uterine fluid from fertile women collected during the MS (receptive) phase (pooled n = 8 women) significantly increased blastocyst outgrowth at 98 h when compared with control treated blastocysts (Fig. 2, B and G). Conversely, addition of uterine fluid from fertile women collected during the MP (nonreceptive) phase (pooled n = 7) significantly decreased outgrowth (Fig. 2, B and I). Furthermore, seven of the 15 blastocysts placed in the fibronectin-coated wells in the presence of MP fluid did not attach; these blastocysts appeared to have degenerated within 48 h of exposure to MP uterine fluid. All other treatment groups had visually healthy blastocysts, of which all attached and spread. Groups of cells within the outgrowth area, particularly those near the edge of the outgrowth appeared to be multinucleated (Fig. 2, D, F, H, and J).

Effect of VEGF and uterine fluid on human endometrial epithelial adhesion

The human endometrial epithelial cell line ECC1 was used to examine the effects of VEGF and uterine fluid on endometrial epithelial cell adhesive capacity. ECC1 cells treated with rhVEGFA (50 ng/ml) showed a greater than 5-fold increase in adhesion to fibronectin (Fig. 3) and a greater than 6-fold increase in adhesion to collagen IV (Fig. 3) in comparison with control (DMEM/TSL with matched BSA concentration). MS-phase uterine fluid likewise increased ECC1 adhesion to fibronectin by more than 2-fold (Fig. 3) and to collagen IV by greater than 5-fold (Fig. 3) when compared with control (matched BSA concentration).

growth. The assay was a measurement of the extent of blastocyst attachment and migration on the extracellular matrix (ECM) component fibronectin. At a dose of 50 ng/ml, rhVEGFA caused the blastocysts to attach and spread over a significantly larger area compared with control (Fig. 2, A, C, and E) ($P < 0.05$ at 66 h after hatching;
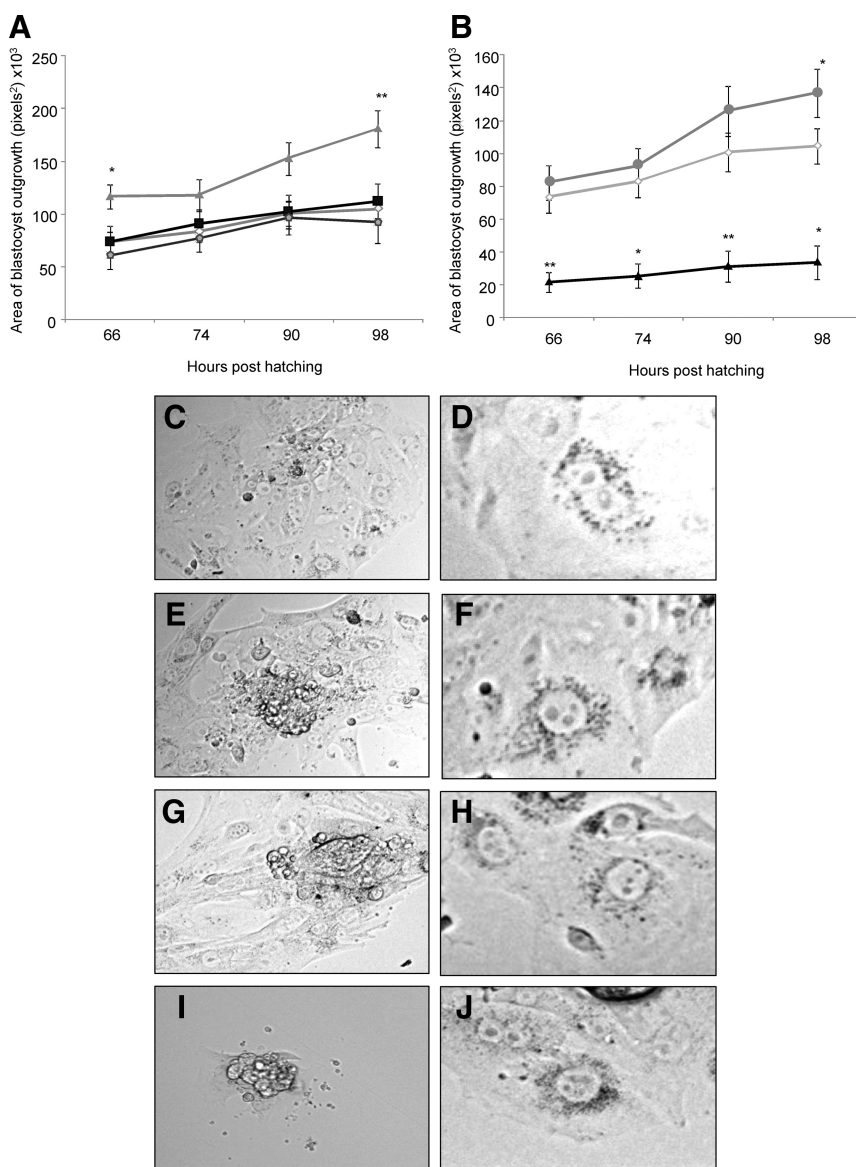
FIG. 2. Blastocyst outgrowth on fibronectin (10 μg/ml) (A) was significantly enhanced with the addition of 50 ng/ml rhVEGF; no significant difference was observed with 5 or 500 ng/ml rhVEGF. (B) Blastocyst outgrowth was also significantly enhanced in the presence of pooled uterine fluid from mid-secretory (MS) phase (n = 8 women), however outgrowth was significantly reduced after exposure to uterine fluid collected during the MP phase. Data are expressed as mean $\pm$ SEM. *, $P < 0.05$; **, $P < 0.01$ (n = 15 blastocysts per treatment). $\diamond$, Control; ■, 5 ng/ml VEGF; ▲, 50 ng/ml VEGF; ⬠, 500 ng/ml VEGF; ●, MS; ▲, MP. Photomicrographs of blastocyst outgrowth on fibronectin substrate (C and D) under control conditions. Enhanced blastocyst outgrowth in the presence of (E and F) VEGF (50 ng/ml) (G and H) and in the presence of uterine fluid from the MS phase and (I and J) reduced outgrowth in the presence of uterine fluid from the MP phase. Magnification, ×20 (C, E, G, and I) and ×100 (D, F, H, and J).

Discussion

Using multiplex analysis, this study identified 30 soluble factors in uterine fluid; eight of these were not previously known. Although considerable variability in the concentration of any one factor was commonly observed between women of proven fertility, even within the MP or MS phase, the concentrations of VEGFA were overall significantly higher in lavage from women of proven fertility than those from women of unexplained infertility. The advantage of this approach was that it enabled simultaneous multiple analyses in a fluid in which many proteins, apart from major serum proteins, are too low in abundance or molecular weight to detect using gel-based proteomic approaches (16, 17). Furthermore, sampling uterine lavage rather than uterine aspirate will include factors that may be bound with low affinity to the surface epithelium and glycocalyx and solubilized by the gentle wash procedure. Functional analysis demonstrated actions of both human uterine fluid and of VEGFA alone on mouse embryo attachment and outgrowth and on endometrial epithelial adhesion to extracellular matrix components present at the maternal-fetal interface.

Cytokines, chemokines, and growth factors are regulatory proteins, produced widely throughout the body by a variety of cell types, and with roles in promoting cell differentiation, migration, and invasion. They act by signaling in both a paracrine and autocrine manner within their local environment. The identification of a number of cytokines and their receptors at the maternal-fetal interface has suggested roles in regulating key events during embryo implantation (26, 27). However, it has not been known whether there are specific changes in the abundance of these factors in the uterine cavity with menstrual cycle phase or fertility status or whether they affect the periimplantation embryo or endometrial receptivity. Importantly, the same cohort of factors are secreted by primary endometrial epithelial cells *in vitro* (28), and therefore, epithelium is likely their major source in uterine fluid, although transudation from blood or release from activated leukocytes cannot be discounted. Furthermore, secretion of six of the factors detected, including VEGFA, is increased in the presence of the blastocyst-derived factor hCG, demonstrating a mechanism by which the presence of a human blastocyst could enhance receptivity (30) and further supporting their importance during
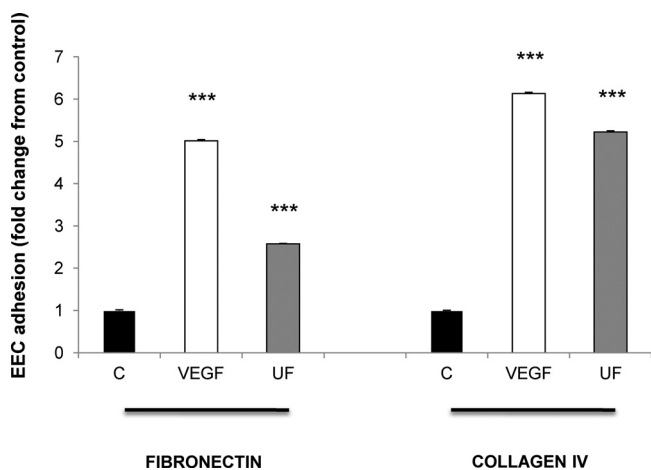
FIG. 3. Endometrial epithelial cell (EEC) adhesion to the trophectoderm ECM components fibronectin and collagen IV. EEC adhesion to both fibronectin and collagen IV was significantly enhanced after treatment with rhVEGF (50 ng/ml). EEC adhesion to fibronectin and collagen IV was also significantly enhanced after culture in the presence of pooled MS-phase uterine fluid (n = 8 women). Results are shown as mean relative adhesion (expressed as fold change from control treated cells) $\pm$ SEM. ***, $P < 0.001$ (n = 15 wells per treatment).

embryo implantation. In the present study, VEGFA levels were significantly reduced in the uterine fluid from women with unexplained infertility compared with fertile women during the MS phase window of implantation, suggesting that VEGFA might be an important soluble mediator during the periimplantation phase.

The current study confirms and extends the results of Boomsma and colleagues (18) who identified a cohort of cytokines in human uterine fluid immediately before embryo transfer. Despite the ovarian stimulation protocol that these women were undergoing during their IVF procedure, many of the same factors (IL-1B, IL-6, IL-10, IL-12, IL-15, CCL2, CCL11, CXCL10, and VEGFA) were identified in uterine fluid in the present study, which examined fluid from natural cycles only. In addition, the current study demonstrated for the first time detection of PDGFAA, TNFB, sIL2RA, FLT3L, sCD40L, IL-7, IFNA2, and CXCL1–3 in human uterine fluid. In contrast to Boomsma and colleagues (15), we did not detect TNFA, IL-17, or IL-5 in any of our samples. These differences may be due to the ovarian stimulation, assay sensitivity, or the different methods (aspiration *vs.* lavage) used to obtain the uterine fluid. Furthermore, Boomsma and colleagues (31) demonstrated a reduced concentration of VEGFA in uterine fluid collected from women after ovarian stimulation when compared with the same women in a natural cycle. Evidence suggests that endometrial receptivity may be impaired by the high doses of hormones used in IVF, and this may compromise chances for successful pregnancy (32, 33), whereas clinical studies have demonstrated lower en-

dometrial and subendometrial blood flow in ovarian stimulated cycles than in natural cycles (34). Here we demonstrate that VEGFA is significantly reduced in uterine fluid collected from women with unexplained infertility compared with fertile women during the MS phase, which is consistent with the Boomsma study and suggests that VEGFA is an important mediator in the uterine cavity during embryo implantation. Furthermore, VEGFA has recently been suggested to be a potential predictive marker for pregnancy success in IVF (35). The study demonstrated that glandular expression of VEGFA in early luteal endometrium was significantly higher in the group that went on to become pregnant when compared with the nonpregnant group. Whether VEGFA alone can predict endometrial receptivity remains to be proven.

The VEGF family and their receptors are key mediators of vascular growth and remodeling in a variety of tissues, including the human endometrium (36). VEGF signaling occurs in a variety of endometrial cells including the vasculature, stroma, and epithelium (36, 37). VEGFA binds and signals via two tyrosine kinase receptors, VEGF receptor 1 (VEGFR1) and VEGFR2, both of which are immunolocalized apically in endometrial glandular epithelium (37). VEGFA can enhance the mitogen activity of endothelial cells via the adhesion molecule integrin $\alpha v\beta 3$ (38), which is an important adhesion molecule during embryo implantation and a potential marker of endometrial receptivity (39, 40). It is produced by the epithelium during the MS phase with specific staining on the apical surface (41), paralleling that of VEGFR1 and -2. Reduced integrin $\alpha v\beta 3$ expression has been observed in endometrium of women with unexplained infertility and thought to account for their recurrent IVF embryo transfer failure (42). Both VEGFR1 and -2 are also expressed by mouse blastocysts (43). Previously, VEGF actions have been demonstrated only on endometrial vasculature. The current study is the first to demonstrate actions of endometrial-derived VEGFA at the maternal-embryonic interface and in particular of secreted VEGFA on both the periimplantation blastocyst and endometrial receptivity. At a dose of 50 ng/ml, rhVEGF caused a significant increase in both blastocyst outgrowth and endometrial epithelial cell adhesive properties on fibronectin, an important ECM component at the forefront of the human maternal-fetal interface (44–45, 29). However, treatment of embryos with doses of 5 and 500 ng/ml VEGF had no affect on blastocyst outgrowth when compared with control. This may be due to saturation of VEGF receptors on the blastocyst at the higher dose or because it may cause a downregulation in receptor expression or secretion of soluble receptors that antagonize VEGF signaling. Additional studies are required to elucidate the precise mechanisms

behind the apparent dose effects observed on embryo outgrowth.

As demonstrated in the current study and by others (15–17), there is a wide array of soluble mediators present in human uterine fluid. The significant outgrowth of the mouse blastocyst when cocultured with MS-phase human uterine fluid demonstrates for the first time a direct effect of these mediators during the periimplantation phase. Given there was no significant difference in the overall abundance or profile of factors present in the uterine cavity during the MS phase when compared with the MP phase, the observation of reduced blastocyst outgrowth and in some instances the complete lack of blastocyst attachment when cocultured with uterine fluid from the MP phase was surprising. This finding suggests the presence of antiembryonic factors during nonreceptive times of the cycle. Previously, we demonstrated significant differences in the abundance of protease inhibitors present in MP- compared with MS-phase uterine fluid (16). This clearly requires further investigation both to identify the factors involved and to confirm the findings with human blastocysts.

Conclusion

This study identified a profile of 30 soluble mediators present in the human uterine cavity across the menstrual cycle, of which VEGFA was significantly reduced in uterine fluid collected during the window of implantation from women with unexplained infertility. Furthermore, functional studies suggest for the first time a direct role for VEGFA and human uterine fluid during the initiation of embryo implantation in the MS phase of the cycle. In contrast, coculture of blastocysts in MP-phase uterine fluid reduced blastocyst attachment and outgrowth. These findings emphasize the concept of precise paracrine and autocrine dialogue between the blastocyst and endometrial epithelium during embryo implantation.

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