Published in: Human Reproduction (2004), vol. 19, iss. 11, pp. 2633-2643
Status : Postprint (Author's version)

Human chorionic gonadotropin and growth factors at the embryonic-endometrial interface control leukemia inhibitory factor (LIF) and interleukin 6 (IL-6) secretion by human endometrial epithelium

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BACKGROUND

The elucidation of the molecular mechanisms by which the embryo contributes to its implantation is an area of extensive research. The main objective of this study was to investigate the pattern of leukemia inhibitory factor (LIF) and interleukin-6 (IL-6) secretion by human endometrial epithelium, and their regulation by human chorionic gonadotropin (hCG) and other growth factors present at the embryonic-endometrial interface. METHODS: Endometrial epithelial cells (EEC) were isolated from biopsies collected at both proliferative and secretory phases of fertile women. RESULTS: HCG (1-50 IU/ml) increased LIF secretion by EEC cultures derived from follicular phase (up to 285 ± 75%) or from secretory phase (up to 212 ± 16%). In contrast, hCG reduced IL-6 secretion by EEC in both phases. The hCG/LH receptor gene was transcribed by EEC as evidenced by RT-PCR. Insulin-like growth factors 1 and 2 increased LIF secretion by EEC. Transforming growth factor β1 stimulated LIF and reduced IL-6 secretion. CONCLUSIONS: Through hCG, the blastocyst may be involved in the control of its implantation (via an increase of proimplantatory LIF) and tolerance (via an inhibition of proinflammatory IL-6). Other growth factors present at the embryonic-endometrial interface are also involved in the control of LIF and IL-6 endometrial secretion.

Keywords: endometrium ; human chorionic gonadotropin ; insulin-like growth factors ; interleukin 6 ; leukemia inhibitory factor ; transforming growth factor beta

Introduction

Implantation of the embryo into the uterine endometrium is a crucial step in the mammalian reproductive process and in the initiation of pregnancy. The success of implantation depends on a cross-talk that is both temporally and spatially synchronized between the blastocyst trophoblast and the endometrial epithelium. An appropriate apposition and close contact between trophoblastic cells and endometrial epithelial cells (EEC) is required for further embryonic development (Giudice, 1999). Human chorionic gonadotropin (hCG) is a specific embryonic glycoprotein, the gene of which (*HCG*) is transcribed as early as the 2-cell stage embryo (Jurisicova *et al.,* 1999). At the blastocyst stage, *HCG* transcripts are detected in trophoblastic cells of the human blastocyst, and hCG production can be detected on day 7 in blastocyst cultures (Dokras *et al.,* 1991; Lopata *et al.,* 1997). Significant levels of hCG may be measured in maternal blood on day 10 after ovulation, close to the time of implantation. In addition to its classical endocrine role as a luteotropic hormone responsible for the maintenance of progesterone production by the maternal corpus luteum, a series of seminal studies have shown that hCG exerts paracrine actions in the uterine environment (Fazleabas *et al.,* 1999; Rao, 2001, 2002). Those studies followed the discovery that receptors for hCG/luteinizing hormone (hCG/LHR) may be labelled by specific antibodies in the human uterus (Reshef *et al.,* 1990). Since then, the expression of hCG/LHR by the human endometrium has been further confirmed through hCG/LHR cDNA studies, radio-binding assays, western blotting, and covalent receptor cross-linking. Treatment of human endometrial glandular epithelial cells with hCG increases the expression of the cyclooxygenase-2 gene (*COX2*) (Zhou *et al,* 1999). When hCG is introduced into the uterine cavity by microdialysis, levels of leukemia inhibitory factor (LIF), vascular endothelial growth factor (VEGF) and metalloprotease 9 (MMP-9) increase in the uterine fluid, while insulin-like growth factor binding protein 1 (IGFBP-1) and monocyte colony-stimulating factor (M-CSF) levels decrease (Licht *et al.,* 2001). In addition, hCG induces morphological and functional differentiation of the endometrial stroma into decidua (Han *et al.,* 1999).

Different cytokines participate in the success of implantation and contribute to endometrial receptivity. Targeted inactivation of *Lif* in mice leads to a complete failure of blastocyst implantation that is rescued after

intraperitoneal LIF administration (Stewart *et al.,* 1992). The role of LIF in human implantation is much less well established. The LIF protein and *LIF* messenger RNA are present in the human endometrium with a maximum level during the implantation window in EEC. At the same time, LIF receptor is expressed by the blastocyst and human endometrium (Charnock-Jones *et al.,* 1994; Arici *et al.,* 1995; Cullinan *et al.,* 1996; Laird *et al.,* 1997). Endometrial cells produce LIF *in vitro* but also *in vivo.* Delage *et al.* (1995a) demonstrated that the *in vitro* secretion by cultured endometrial expiants obtained from infertile women were significantly lower when compared to fertile women. Laird *et al.* (1997) further confirmed that the amount of LIF in flushings obtained from women with unexplained infertility was significantly lower than those from normal fertile women on day LH + 10. On the contrary, when measured on day 26 of the cycle, a low concentration of LIF in uterine flushing is predictive of subsequent implantation. An excess of LIF in the luminal fluid at this time could reflect local inflammation which is detrimental for implantation (Ledee-Bataille *et al.,* 2002). Interleukin 6 (IL-6) is also secreted in the human endometrium following a temporal profile during the menstrual cycle, with levels rising during the luteal phase and menstruation (Tabibzadeh *et al.,* 1995; Vandermolen and Gu, 1996). The IL-6 receptor is expressed at the surface of the embryo, the trophoblast and the endometrium (Sharkey *et al.,* 1995). Blastocyst implantation is not impaired in *Il6*$^{-/-}$ mice, although the development of the conceptus is compromised (Robertson *et al.,* 1992; Kopf *et al.,* 1994; Salamonsen *et al.,* 2000).

The main objective of this study was to further explore the hypothesis that hCG, and growth factors present at the embryonic-endometrial interface and known to intervene in embryo development (IGFs and TGF-β1), are able to control LIF and IL-6 secretion by human EEC.

Materials and methods

Patients

Endometrial biopsies were collected from fertile women (one previous delivery) with normal cycles (n = 28) undergoing surgery for voluntary sterilization or during hysteroscopy before assisted medical procreation (AMP) because of male infertility. The stage of the menstrual cycle was established from women's menstrual history (based on the first day of the last menstruation) and from histological dating performed by experienced pathologists, according to the criteria of Noyes *et al.* (1950). Fourteen patients were in the proliferative phase (group A) and fourteen in the secretory phase (group B). The mean age of group A was 33.5 years (range 18-45 years) and the mean age of group B was 35 years (range 26-46 years). None of the patients received any hormonal treatment for 3 months prior to biopsy. The ethics committee of Liege Medical School approved the protocol, and informed consent was obtained from the patients.

Separation of endometrial epithelial cells from stromal cells

Endometrial biopsy samples were collected in Hanks' balanced salt solution (HBBS) containing penicillin (100 IU/ml) and streptomycin (100 µg/ml) and endometrial epithelial cells (EEC) were isolated following the method described by Laird *et al.* (1993). The tissues were finely chopped with scissors and digested for 45 min at 37°C in 5 ml of Dulbecco's modified Eagle's medium (DMEM) containing 0.2% collagenase type la (Sigma Aldrich, St Louis, MO). During incubation and at the end of incubation, the tissue was gently pipetted every 15 min to disperse the cells. The EEC were separated from stromal cells by centrifugation for 20 min at 100 *g*. Pellets contained mainly epithelial cells present both as small clumps and as separated cells. The two withdrawn supernatants, containing mainly stromal cells, were pooled and centrifuged at 300 *g* to pellet the cells. Epithelial and stromal cells were purified by unit density sedimentation. Each fraction of cells was resuspended in 2 ml DMEM containing 10% fetal calf serum (FCS), 1% glutamine and antibiotics, and gently pipetted onto 8 ml of DMEM + 10% FCS in a test tube and left for 30 min at room temperature. Afterwards, epithelial cells were in the lower 2 ml, while stromal cells were in the upper 8 ml of supernatants. Epithelial cells were used for immunocytochemistry, cell culture/cytokine production and for molecular biology. Before culturing in serum-free medium, epithelial cells were centrifuged at 100 *g*, and the pellets were washed three times with DMEM containing 1% glutamine, antibiotics, 0.3% bovine serum albumin and 25 mIU/ml human insulin. Stromal cells were used only for immunocytochemistry.

Cell cultures

EEC were plated into 24-well plates at 2×10^5 cells per ml and per well and cultured for 72h in DMEM with 1% glutamine, 0.3% bovine serum albumin and 25 mIU/ml human insulin. Each treatment was applied in a micro volume (50 µl) at the beginning of incubation. Each condition was tested in triplicate. Too few EEC after separation resulted in the exclusion of the expiant from some treatments. The cells used for immunocytochemistry were cultured for 24 h on multi-test slides in media containing DMEM with 1% glutamine, 25 mIU/ml human insulin and 10% FCS.

Cytokines, growth factors and antibodies

Recombinant (rec) IL-1β was provided by Preprotech (London, UK) and used at a final concentration range between 10^{-9} and 10^{-12} M. Rec IL-1α was obtained from Genzyme (Cambridge, UK) and was used at 10^{-9} M. Anti-human IL-lβ antibody was provided by R&D Systems (Abingdon, UK) and used at a concentration of 50 μg/ml. Phytohemagglutinin (PHA) was provided by Glaxo-Wellcome (Zebulon, USA) and used at a final concentration of 5 μg/ml. Lipopolysaccharide (LPS) was purchased from Sigma and used at a final concentration of 20 μg/ml. Human chorionic gonadotropin (hCG) (Pregnyl) was provided by Organon (Brussels, Belgium) and was added at a final concentration of 1, 5, 10 and 50 IU/ml. Anti-hCG antibody from Santa Cruz Biotechnology (Santa Cruz, USA) was added at a final concentration of 2 μg/ml. Transforming growth factor-β1 (TGF-β1) was prepared according to the methodology of Cone *et al.* (1988) and used at 10^{-8}, 10^{-10} and 10^{-12} M.
Human insulin-like growth factor (IGF)-1 and IGF-2 (R&D Systems) were used at three final concentrations: 10^{-8}, 10^{-10} and 10^{-12} M.

Anti-human cytokeratin (5, 6, 8, 17 and 19) mAb (clone MNF 116, code no. M0821) and anti-vimentin V9 were from Dako (Copenhagen, Denmark). mAb anti-CD45RB was obtained from Dako. Second antibodies (Jackson, Pennsylvania, USA) were fluorescein (FITC)-conjugated goat anti-mouse IgG (second antibody A) and rhodamine (TRITC)-conjugated goat anti-rabbit IgG (second antibody B).

Cytokine immunoassays

Supernatants of cultured EEC were collected for assay after 72h. Collected media were stored at -20°C until cytokine measurements. All cytokine (IL-6 and LIF) concentrations were measured with specific ELISAs (Biosource-Europe, Nivelles, Belgium) according to appropriate instructions (De Groote *et al.,* 1992). Sensitivity was 20pg/ml for LIF and 3pg/ml for IL-6. Range was 150-8200pg/ml for LIF and 8.5-540pg/ml for IL-6. Inter and intra-assay coefficients of variation (CV) for LIF were <8.4% and <5%, respectively. Inter and intra-assay CV for IL-6 were <9.3% and <7.7%. For LIF assays, results obtained were usually in the standard curve and a preliminary dilution was not necessary. When some rare results were not in the standard curve, a new assay was performed with diluted supernatants. For IL-6 assay, the supernatants were usually diluted (systematically for the IL-1β condition) to ensure that they were in the limits of the standard curve.

Proliferation test

Proliferation tests were performed on EEC cultured for 72h from three different endometria, using a 5-bromo-2'deoxyuridine (BrdU) cell proliferation colorimetric immunoassay provided by Roche, according to the manufacturer's instructions.

Immunocytochemistry

Multitest slides were kept for 24h in FCS-epithelial or stromal cell medium to allow cell plating (5000 cells/well) and then stained after -20°C methanol fixation (Robert *et al.,* 1991; Martens *et al.,* 1996). Slides were first incubated with 10% normal goat serum to prevent non-specific binding of goat second antibody to mouse or rabbit Ig. Immunostaining was performed with the following reagents: anti-human cytokeratin mAb MNF 116 diluted at 1:50, anti-vimentin V9 mAb diluted at 1:500, or anti-CD45RB mAb diluted at 1:100, followed by second antibody A or B. The slides were mounted with glycerol/gelatin medium and observed using an Olympus fluorescence microscope. Images were collected with 20× and 40× objectives. A mercury source was used for fluorescein (488 nm) and rhodamine (568 nm).

Molecular biology

RNA extraction

EEC samples isolated from seven different endometrial biospies were frozen in liquid nitrogen (or at -70°C). Total RNA was extracted using an RNeasy Mini Kit from Qiagen (Valencia, USA) according to the manufacturer's instructions. Samples were treated by RNase free DNase (Roche, Indianapolis, USA) to remove potentially contaminating DNA. The integrity of extracted RNA was assessed by electrophoresis in ethidium bromide-stained 1% agarose MOPS/EDTA gel.

RT-PCR

Two hundred and fifty nanograms of total RNA were subjected to reverse-transcriptase using First Strand cDNA Synthesis Kit (Roche). In this method, RNA is reverse transcribed by AMV into single-stranded cDNA. The cDNA was secondarily amplified by FastStart Taq DNA Polymerase (Roche) using the primers corresponding to human hCG/LHR cDNA sequence (upper primer 5'-GGCCGAAAACCTTGGATATTT-3', position 676-696; and lower primer 5'-GTAAGTCAACGTGGCCTCCAG-3', position 813-833). The expected PCR product was 158 bp in length. Human β-actin PCR primer pair (R&D Systems, Abingdon, UK) was used, resulting in a PCR product of 528 bp. Fifty microlitres of PCR reaction mixture contained 20 pmol of each oligonucleotide primer

and 2 IU of Taq DNA polymerase. The reaction was started at 94°C for 5 min and run for 40 cycles (45 sec at 94°C, 45 sec at 61°C and 1 min at 72°C). Aliquots of 15 µl of amplified fragments were visualized in ethidium bromide-stained 2% agarose gel. The size of DNA species generated was determined using EZ load precision Molecular Mass Standard (Bio-Rad, Hercules, USA) and a 100 bp ladder (Pharmacia Biotech, Uppsala, Sweden). RT-PCR products were sequenced using ABI prism BigDye Terminator Cycle Sequencing Ready Reaction Kit protocol (Genomexpress, Meylan, France) to ensure *hCG/LHR* identity.

Mathematical and statistical analyses

Because of individual variability, all data have been normalized and expressed as the percentage of the mean of basal values (mean ± SEM). For each experiment (except when specified), statistical analysis of the response to any kind of treatment was performed for the difference with basal value. The significance of differences was evaluated using unpaired nonparametric Mann-Whitney test with confidence intervals of 95%. Statistical analyses were performed using GraphPad Prism software.

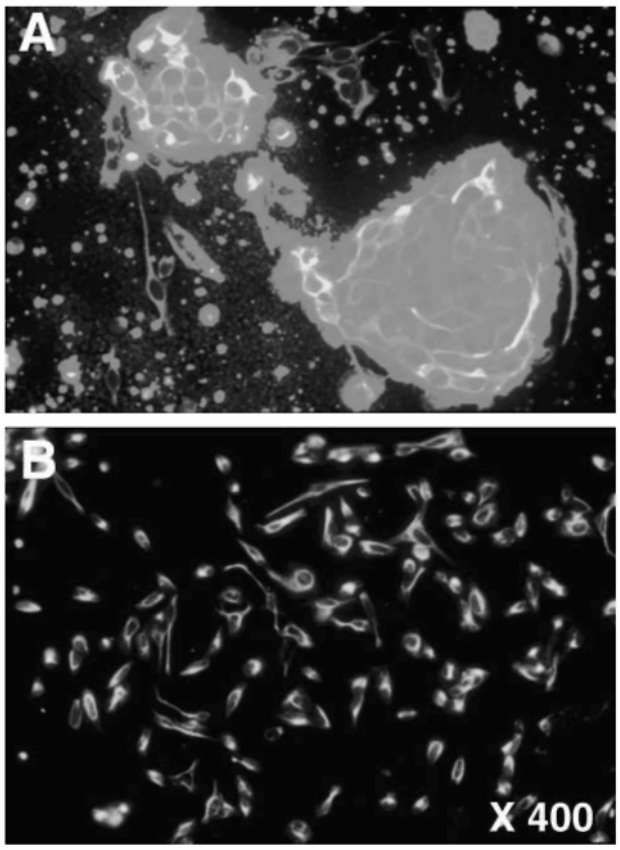
Results

Characterization of cell cultures

Immunocytochemistry

The epithelial nature of the cells pelleted after 100 *g* centrifugation was demonstrated by their intense staining with a mAb against human cytokeratin MNF 116 (Figure 1A). These cells were not stained either by a mAb against vimentin, or by a mAb staining all white blood cells (anti-CD45RB) (data not shown). Cytokeratin-positive epithelial cells represented >95% of the total cell population. By contrast, >95% of the cells in the supernatants were vimentin-positive and cytokeratin-negative stromal cells (Figure 1B). Results were similar without serum addict, with restriction of lower cell adhesion (data not shown).

Figure 1. *(A) Immunostaining of the epithelial fraction of human endometrium with mAb anti-cytokeratin MNF 116, 1:50. (B) Immunostaining of the stromal fraction of human endometrium with mAb anti-vimentin V9, 1:500.*



Published in: Human Reproduction (2004), vol. 19, iss. 11, pp. 2633-2643
Status : Postprint (Author's version)

Cytokine production

Under basal conditions, cultured human EEC spontaneously secreted LIF and IL-6 (Table I). This production increased in a time-dependent way with a maximum level after 3 days of culture (data not shown). A 72h incubation was therefore selected for all further experiments. The addition of LPS (20 µg/ml), PHA (5 µg/ml), or a combination of both did not induce any significant increase in cytokine secretion compared to the basal condition (Figure 2A).

The addition of a small amount of IL-1β (10^{-9} M) markedly increased LIF secretion. This stimulating effect of IL-1β was already observed at 10^{-12} M and increased progressively from 10^{-12} M to 10^{-9} M (Figure 2B). A response of the same amplitude was observed with IL-1α at 10^{-9} M. IL-1β also induced a strong concentration-dependent increase of endometrial IL-6 secretion (Figure 2B). Consequently, treatment with IL-1β (10^{-9} M) was used as positive control in all cultures.

Table I. *Mean of basal productions (pg/ml) of LIF and IL-6 by EEC from all proliferative and secretory endometria*

	Phase	Basal production (pg/ml)	Standard error	*n*	Mann -Whitney test
LIF (pg/ml)	Proliferative	320.9	45.29	14	*P* = 0.7 (NS)
	Secretory	331.9	42.29	14	
IL-6 (pg/ml)	Proliferative	272.4	39.9	14	*P* = 0.07 (NS)
	Secretory	522.3	92.6	14	

Figure 2. (A) *Production of LIF and IL-6 by 72h cultures of human EEC under basal conditions, and after treatment with PHA (phyfhohemagglutinin), LPS (lipopolysaccharide), the combination of both, and IL-1β (10^{-9} M). n = 3 (proliferative and secretory phases pooled). Results are expressed as mean ± standard error of percentage of basal. ***P < 0.001; NS = not significant by Mann-Whitney test. (B) Production of LIF and IL-6 by 72h cultures of human EEC under basal conditions, and after treatment with IL-1β (10^{-12} M to 10^{-9} M) and IL-1α (10^{-9} M). n = 4 (proliferative and secretory phases pooled). Results are expressed as mean ± standard error of percentage of basal. ***P < 0.0001; NS = not significant by Mann-Whitney test.*

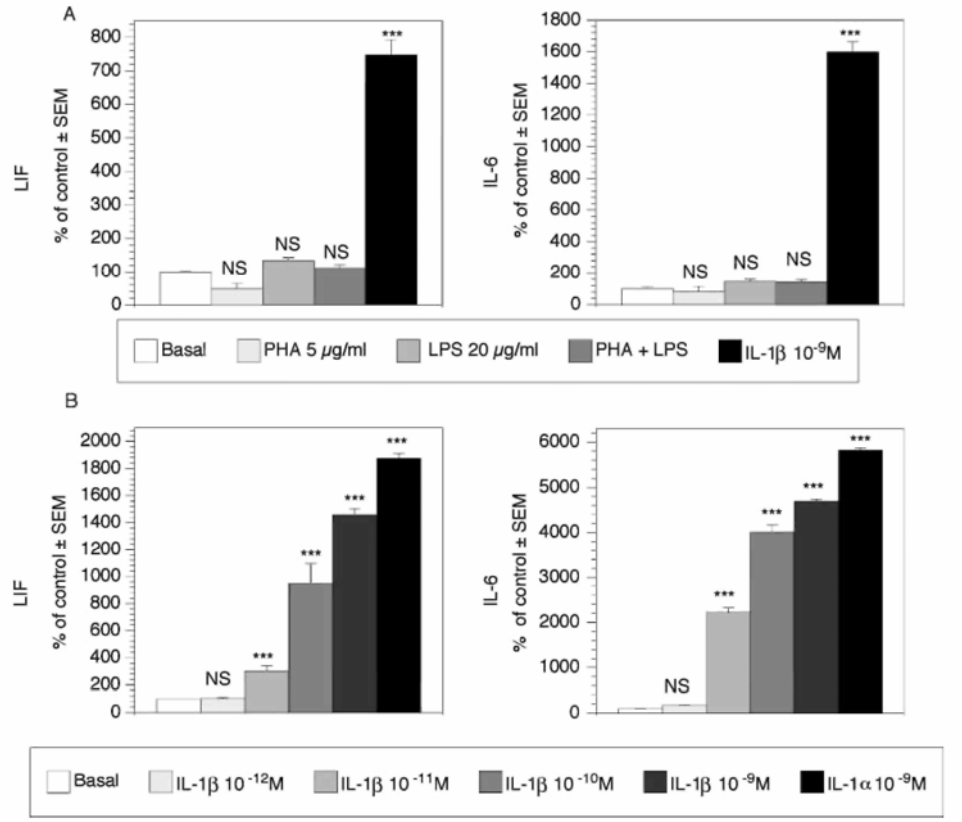


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Table II. *Absorbance values (mean ± standard deviation) after BrdU proliferation test by ELISA*

N = 3	Absorbance (mean ± St.dev.)	Mann-Whitney test
Basal	0.10 (±0.02)	NS
hCG 50 IU/ml	0.10 (±0.02)	NS
IGF1 10^{-8} M	0.13 (±0.05)	NS
IGF2 10^{-8} M	0.13 (±0.03)	NS
TGF-β1 10^{-8} M	0.09 (±0.06)	NS
IL-1β 10^{-9} M	0.14 (±0.04)	NS
EGF	0.22 (±0.07)	$P = 0.02$
FCS 10%	0.25 (±0.05)	$P = 0.016$

Statistical analyses performed using Mann-Whitney test.
NS — not significant.

Regulation of endometrial cytokine production by hCG, IGFs and TGF-β1

Results were analysed according to the phase of the menstrual cycle: group A for proliferative phase and group B for secretory phase. The proliferative effect of the different reagents tested on EEC cultured for 72h was assessed in triplicate by a BrdU cell proliferation test provided by Roche (Table II). No significant proliferative effect was found for hCG (50 IU/ml), IGF-1 (10^{-8} M), IGF-2 (10^{-8} M), TGF-β1 (10^{-8} M) or IL-1β (10^{-9} M).

Effect of hCG

In both groups, hCG at concentrations ranging between 1 and 50 IU/ml stimulated the secretion of LIF by EEC in a concentration-dependent manner after 72h (Figure 3A). This stimulating effect of hCG (50 IU/ml) was inhibited by a neutralizing anti-hCG antibody (2μg/ml) (Figure 3C). This effect of hCG on LIF production was not mediated by IL-1β since a blocking antibody to IL-1β did not modify the response to hCG (Figure 3D). Interestingly, hCG exerted an opposite effect on IL-6, reducing its production in both groups (Figure 3B). This effect was also inhibited by the neutralizing anti-hCG antibody (2 μg/ml) (Figure 3C). Statistical analyses did not reveal any significant difference in hCG effects between the two groups.

Effect of IGF-1 and IGF-2

IGF-1 (Figure 4A) and IGF-2 (Figure 4B) induced a concentration-dependent increase of LIF production by endometrial epithelial cells after 72h, but did not exert any significant effect on IL-6 production. All EEC from both secretory and proliferative phases responded similarly to IGF-1 and IGF-2 with regard to LIF and IL-6 secretion.

Effect of TGF-βl

In both groups, TGF-β1 induced a concentration-dependent increase of LIF secretion, and a concentration-dependent decrease of IL-6 secretion (Figure 5). No significant difference was found between the two groups.

Expression of human hCG/LHR in human endometrium

As shown in Figure 6, transcripts of the *hCG/LHR* gene were evidenced by RT-PCR analyses performed on EEC isolated from endometria either in proliferative or in secretory phase. These results have been repeated and confirmed on five other different EEC samples. Human granulosa cells were used as positive control. RT-PCR products were sequenced and were found to correspond with the *Homo sapiens* luteinizing hormone receptor (gi 4557716).

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Figure 3. (A) *Production of LIF by EEC in proliferative phase (n = 9) and secretory (n= 12) phase under basal conditions and after treatment with hCG (1-50 IU/ml) expressed as mean ± standard error of percentage of basal. The LIF response to IL-1β is shown as positive control. *P = 0.004, **P < 0.0001, NS = not significant by Mann-Whitney test. (B) Concentration-dependent effect of hCG (1-50 IU/ml) on IL-6 production by human EEC in proliferative phase (n = 9) and in secretory phase (n = 12). The IL-6 response to IL-1β is shown as positive control. Results are expressed as mean ± standard error of percentage of basal. *P = 0.002; **P = 0.0008; ***P < 0.0001 by Mann-Whitney test. (C) Effect of hCG (50 IU/ml) on LIF and IL-6 production by human EEC. A neutralizing antibody against hCG (2 µg/ml) inhibits the LIF and IL-6 responses to hCG (50 IU/ml). n = 6 (proliferative and secretory phases pooled). Results are expressed as mean ± standard error of percentage of basal. **P = 0.0004; ***P < 0.0001; NS = not significant (vs basal); ♦P < 0.0001, ♦♦P = 0.0004 (vs hCG 50 IU/ml) by Mann-Whitney test. (D) Concentration-dependent effect of hCG (5-50 IU/ml) on LIF production by human EEC. A blocking antibody against IL-1β (50 µg/ml) does not modify the LIF response to hCG (50IU /ml). n = 4 (proliferative and secretory phases pooled). Results are expressed as mean ± standard error of percentage of basal. *P = 0.002; NS = not significant (vs basal); NS = not significant (vs hCG 50IU /ml); ♦♦♦P = 0.002 (vs IL-1β 10^{-9} M) by Mann-Whitney test.*

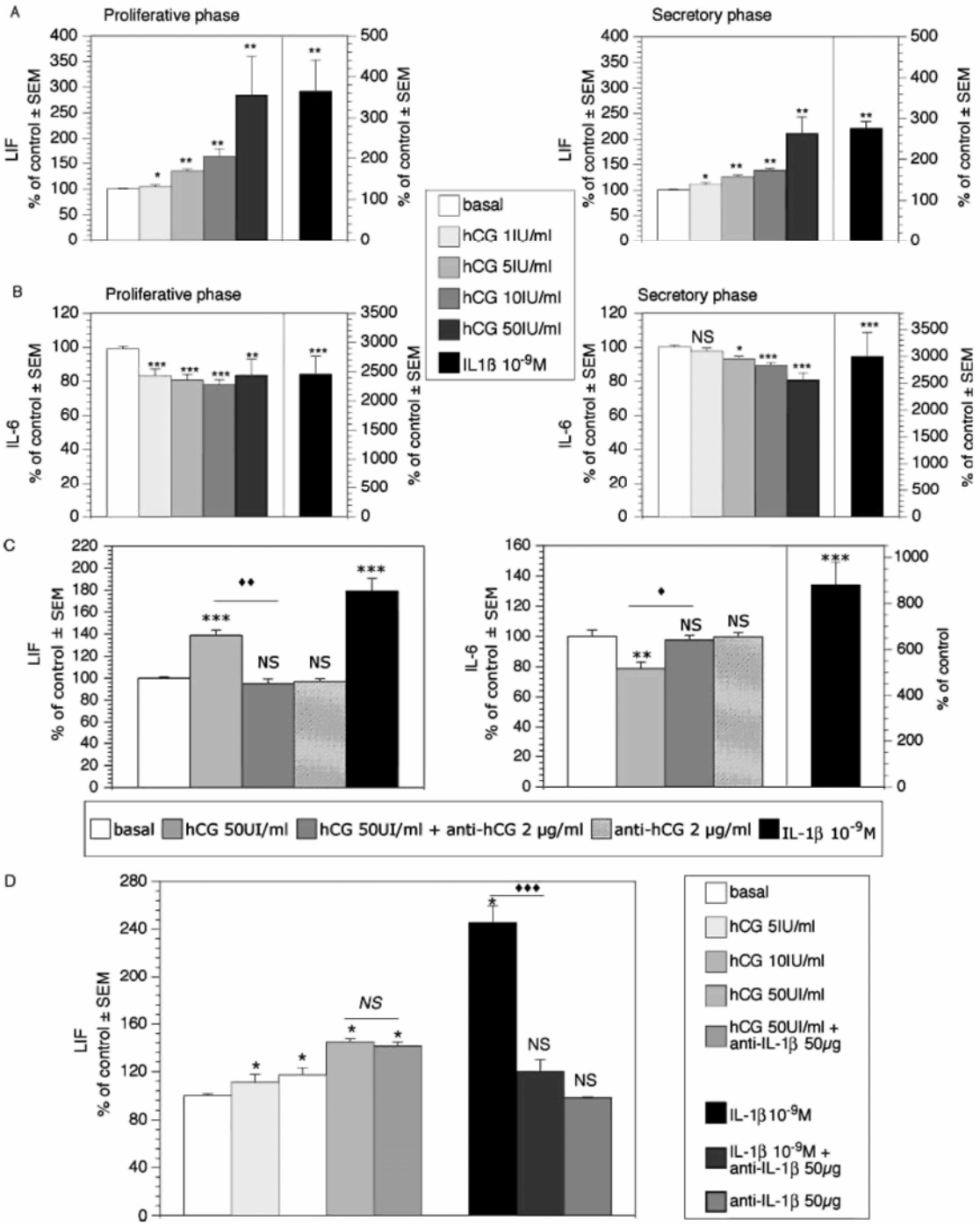

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Figure 4. (A) *Effect of IGF-1 (10^{-12} M to 10^{-8} M) on LIF and IL-6 production by 72h cultures of human EEC in proliferative phase (n = 6) and in secretory phase (n = 9). Results are expressed as mean ± standard error of percentage of basal. (B) Effect of IGF-2 (10^{-12} M to 10^{-8} M) on LIF and IL-6 production by 72h cultures of human EEC in proliferative phase (n = 6) and in secretory phase (n = 9). Results are expressed as mean ± standard error of percentage of basal. *P = 0.02; **P = 0.006; ***P = 0.0002; ****P < 0.0001; NS = not significant by Mann-Whitney test.*

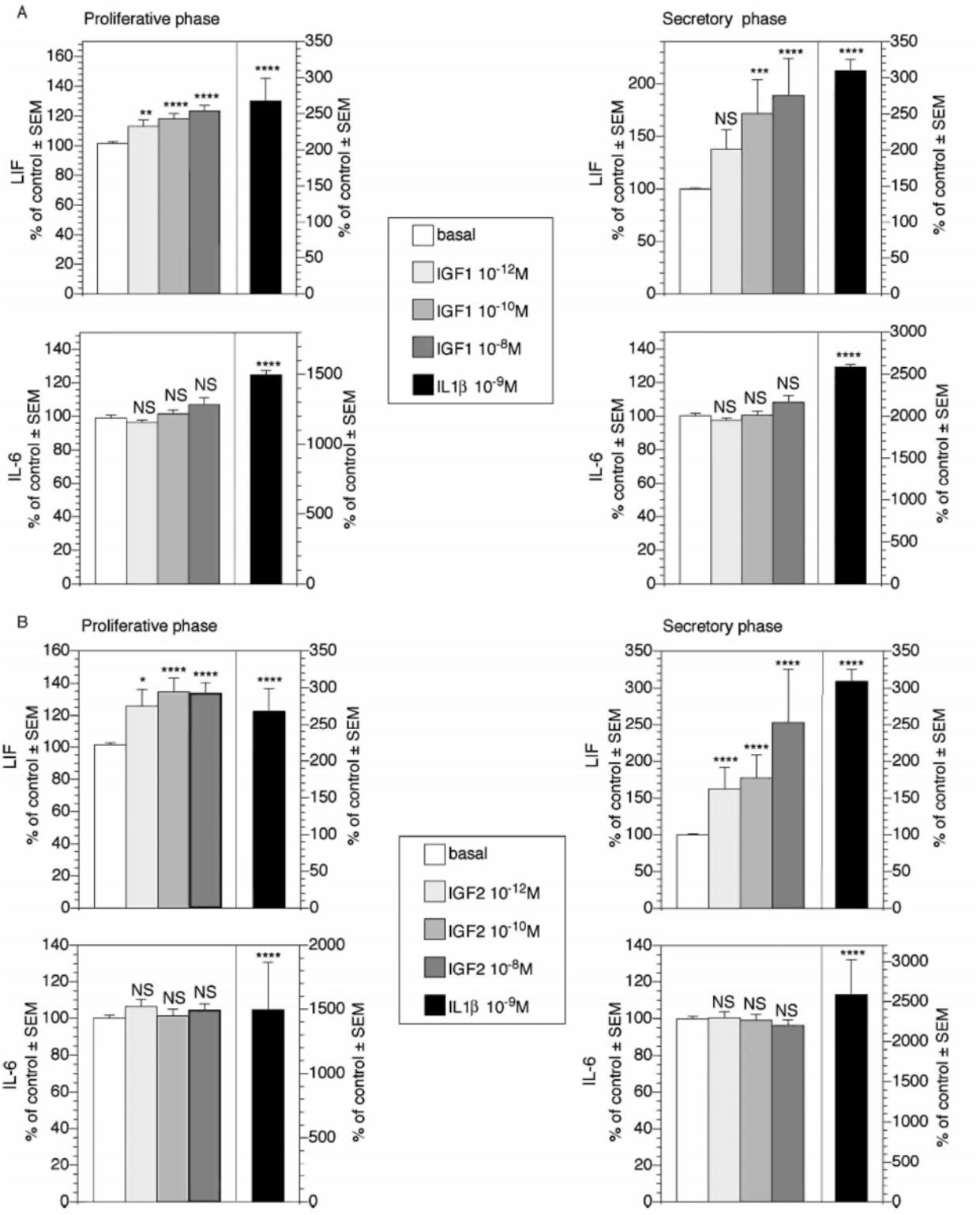

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Figure 5. *Effect of TGF-β1 (10^{-12} M to 10^{-8} M) on LIF and IL-6 production by 72h cultures of human EEC in proliferative phase (n = 10) and in secretory phase (n = 8) expressed as mean ± standard error of percentage of basal. The LIF and IL-6 responses to IL-1β are shown as positive control, ***P < 0.0001 by Mann-Whitney test.*

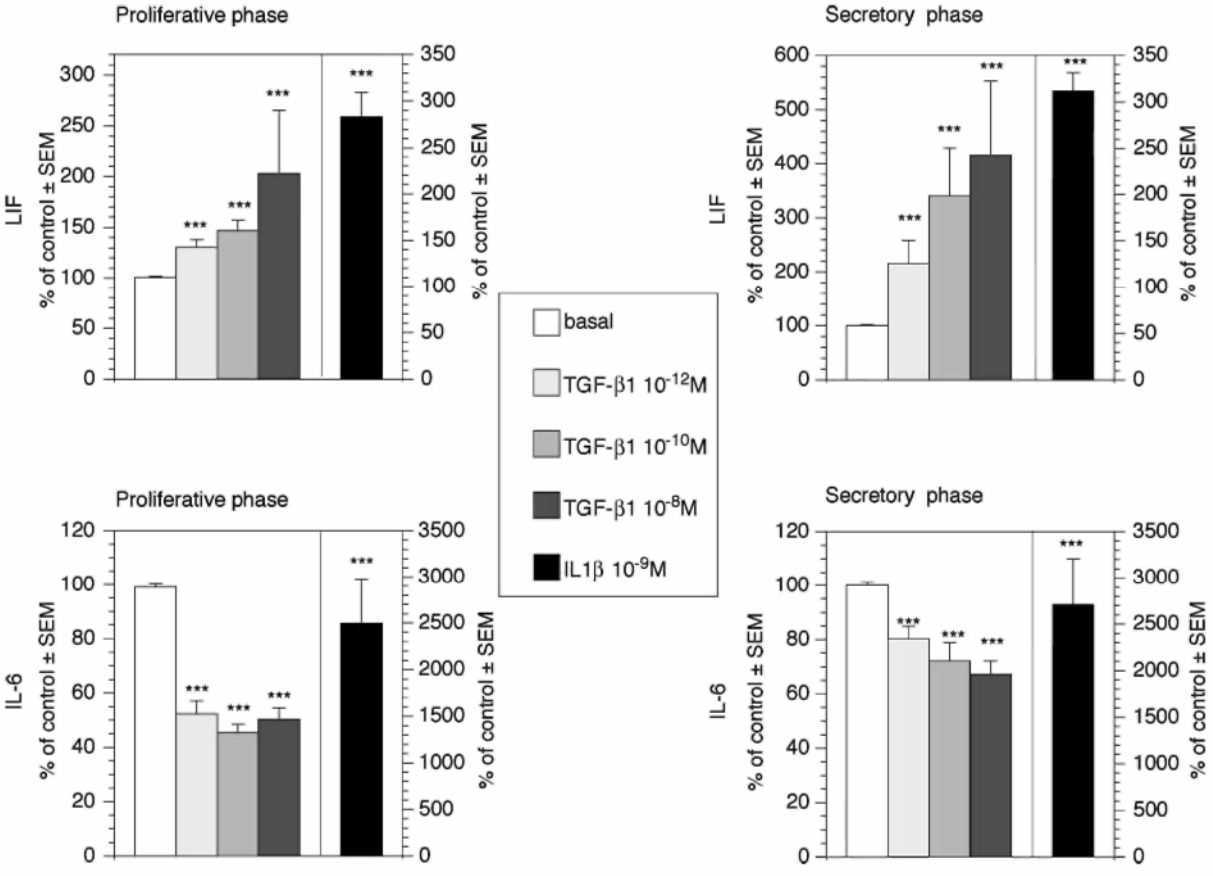
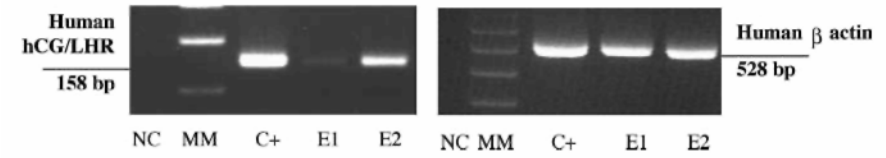


Figure 6. *Detection by RT-PCR of human hCG/LHR gene transcripts. Left: amplification of cDNA obtained with hCG/LHR-specific primers (158 bp). Right: amplification of cDNA obtained with β-actin specific primers as internal control (528 bp). MM: EZ load precision molecular mass standard. NC: H_2O (negative control). C + = granulosa cells; E1 = EEC from an endometrium in proliferative phase; E2 = EEC from an endometrium in secretory phase.*



Discussion

The epithelial nature of the pelleted endometrial cells was confirmed by their intense staining with a mAb against cytokeratin, and by the absence of any staining with a mAb against vimentin as well. The absence of contaminating leukocytes was shown by the failure of any cell labelling by a mAb anti-CD45RB and was confirmed by the incapacity for LPS or PHA (or both) to modify LIE and IL-6 secretion by EEC. Human endometrial epithelium spontaneously secreted LIE and IL-6 in proliferative and in secretory phase. When the basal production of all the biopsies in proliferative phase or in the secretory phase were pooled, there were no statistically significant differences between proliferative phase and secretory phase. These results are not in contradiction with the *in vivo* profile of LIF (increased in late proliferative and early secretory phase) or IL-6 (increased in secretory phase) productions by EEC. Indeed, our results did not exclude that, for any patient, its basal production of LIF or IL-6 is higher in the secretory phase compared to the proliferative phase. Moreover, our results concerning LIF basal production are not inconsistent with those of Laird *et al.* (1997) since we did not subdivide the phases in early, mid and late proliferative/secretory phase as those authors did. The basal

production of LIF and IL-6 follows a time-dependent pattern, the maximum being reached after 3 days of culture. Thus, a 72h incubation was selected for all further experiments. IL-1β 10^{-9} M strongly stimulated both LIE and IL-6 secretion by human EEC and was used as positive control in further experiments. We further extend previous results obtained for LIF by Arici *et al.* (1995) on stromal cells and Delage *et al.* (1995b) and confirm the results obtained by Laird *et al.* (1994) and Vandermolen and Gu (1996) for IL-6. Since the degree of cytokine response significantly varied from one woman to another, all the cytokine data were normalized (see Materials and methods). None of the factors tested at the highest concentration could induce a proliferative effect as tested with a BrdU cell proliferation ELISA.

LIF is known to stimulate hCG production by trophoblast (Nishino *et al.,* 1990; Sawai *et al.,* 1995). In our cultures of human EEC, hCG stimulated LIF production in a concentration-dependent way, suggesting that the blastocyst may influence its own implantation through hCG This positive effect of hCG on LIF production was specific since it was blocked by a neutralizing anti-hCG antibody and not mediated by a local action of IL-1β since it was not blocked by an anti-IL-1β antibody. Our results also confirm and add to the *in vivo* results of Licht *et al.* (2001) who observed an increase of LIF levels in uterine fluids after microdialysis of the uterine cavity with hCG. The expression of *hCG/LHR* by human endometrial epithelium in proliferative and secretory phase was evidenced by RT-PCR. Some controversy about the classical *LH/hCGR* expression in the human endometrium was raised by Stewart *et al.* (1999) and Stewart (2001). Several groups have however shown the presence of hCG/LHR in extragonadal human, rabbit or mouse female reproductive organs (Ziecik *et al.,* 1986; Jensen and Odell, 1988; Bonnamy *et al.,* 1990; Reshef *et al.,* 1990; Bhattacharya *et al.,* 1993; Bonnamy *et al.,* 1993; Derecka *et al.,* 1995; Zhang *et al.,* 2001). Moreover, using nested RT-PCR, Licht *et al.* (2003) recently demonstrated the expression of full-length *hCG/LHR* mRNA in human endometrium regulated by alternative splicing. In our study, we detected *hCG/LHR* transcripts on EEC by non-nested RT-PCR assays. Despite not observing an effect of cycle stage on the ability of hCG to regulate LIF and IL-6 in EEC cultures, the *hCG/LHR* expression seems to be lower in the proliferative phase compared to the secretory phase. Further Real Time RT-PCR experiments are necessary to exactly quantify the amount of *hCG/LHR* expression throughout the cycle. As suggested by Fazleabas *et al.* (1999) in the *Papio anubis* endometrium, our results confirm that hCG, besides its endocrine role in maintaining progesterone production by the corpus luteum, also exerts a paracrine action on the human endometrial epithelium.

Previous studies have shown that IL-6 interacts with IL-6 receptor on the trophoblast, resulting in hCG release (Nishino *et al.,* 1990; Masuhiro *et al.,* 1991; Li *et al.,* 1992). Our results show that hCG interacts with hCG/LH-R on EEC, resulting in IL-6 inhibition. Further experiments are necessary to elucidate whether hCG could also have such a negative feed-back on IL-6 secretion by trophoblast. Through the inhibition of the pro-inflammatory endometrial IL-6, hCG may play a new role as an immune regulator at the embryonic-endometrial interface during implantation. In our cultures of purified EEC from 28 fertile women, we did not obtain the same results as Uzumcu *et al.* (1998) who used a mixed population of endometrial cells from six IVF patients, and who observed that hCG (100 and 500 IU/ml) stimulated IL-6 production. The role of hCG as immunosuppressive factor was already suggested by its inhibition of PHA- or con-canavalin A-mediated proliferation of human lymphocytes *in vitro.* Moreover, hCG inhibits IL-2 and simultaneously stimulates sIL-2R (a soluble form of the IL-2R which modulates IL-2 bioavailability) secretion by cultures of human peripheral blood mononuclear cells, suggesting a direct role for hCG in regulation of lymphocyte biological activity (Nakayama *et al.,* 2002). Khan *et al.* (2001) also confirmed the regulatory role of hCG on Th1 cell activity since the treatment of non-obese diabetic (NOD) mice with hCG before the onset of clinical symptoms dramatically inhibited the development of diabetes for a prolonged period of time. Transfer of spleen cells from the hCG-treated NOD mice into immunodeficient NOD/SCID mice blocked the development of diabetes in these mice (Khan *et al.,* 2001). Finally, recent results of Kayisli *et al.* suggested that hCG could be a link in the development of maternal immunotolerance and may facilitate trophoblast invasion by regulating proapoptotic molecules such as Fas-Fas ligand system in endometrial cells (Kayisli *et al.,* 2003).

IGFs induced a marked increase of LIF secretion without any significant effect on IL-6. In the endometrium, IGFs and their binding proteins (IGFBPs 1-6) are derived from stromal cells, whereas the epithelium predominantly expresses IGF receptors (IGF type 1 receptor or IGF-1R, IGF type 2 receptor or M6P/IGF-2R) (Rutanen, 1998). IGFs and predominantly IGF-2 are also detected in many fetal tissues from the first trimester (Han and Carter, 2000). In mouse embryos, increasing *Igf1* mRNA levels are observed between the 8-cell and blastocyst stages (Doherty *et al.,* 1994), while *Igf2* expression has been detected from the 2-cell stage. In addition to a paracrine/autocrine control of the cycling endometrial development, the IGFs and their binding proteins appear to play a crucial role during implantation and establishment of pregnancy (Nayak and Giudice, 2003). It is increasingly evident that disturbances in the IGF system occur in complications of human pregnancy, such as intrauterine growth restriction, as well as pre-eclampsia (Irwin *et al.,* 1999). In our experimental model, we show that, in the presence of IGF-1 and IGF-2, pro-implantatory LIF production by EEC is increased *in vitro.* The *in vivo* source of IGF-1 and -2 might be endometrial stromal cells, but also the pre-implantatory embryo.

Indeed, *IGF1* and *IGF2* mRNA are expressed by human trophoblast, IGF-2 being the most abundantly expressed.

The TGF-β1 isoform markedly enhanced LIF secretion while, like hCG, it reduced IL-6 release by cultured epithelial cells from the human endometrium. The TGF-β superfamily related glycoproteins are well known for their role in the induction of embryonic mesoderm and also for their immunosuppressive properties. In mammals, there are four isoforms of TGF-β: TGF-β1, -β2, -β3 and -β4 (Tabibzadeh *et al.,* 1998). The TGF-β superfamily involves many members, such as bone morphogenic proteins, inhibins, activins and Müllerian inhibitory factor. TGF-β is known to induce *in vitro* cytotrophoblast differentiation into syncytiotrophoblast, controlling then the process of placentation. The four isoforms of TGF-β are present in the endometrial epithelium and stroma throughout the menstrual cycle in a time-dependent manner, their expression being the highest in the secretory phase (Kauma *et al.,* 1990; Casslén *et al.,* 1998; Godkin and Doré, 1998; Tabibzadeh *et al.,* 1998). Our data show that, besides its mesoderm-inducing properties within the embryo, TGF-β1 may also contribute both to implantation by enhancing endometrial LIF production, and to maternal tolerance of the fetal graft by inhibiting IL-6 release. This tolerogenic action of the TGF-β superfamily had already been suggested by expression of a TGF-β2-related molecule in the murine decidua (Clark *et al.,* 1995; Merali *et al.,* 1996). Our results are in line with Arici *et al.* (1995) who demonstrated that TGF-β and IL-1β are potent inducers of LIF expression in endometrial stromal cell culture.

Although IL-6 and LIF signal through the same molecule gp 130, they may exert different effects in endometrial cells, suggesting the activation of different intracellular signalling pathways, which may ultimately be important in the control of endometrial function (Ernst and Jenkins, 2004). This was also suggested by Cork *et al.* (2001) on epithelial and stromal cells since LIF and ΓL-6 had no effect on tumour necrosis factor (TNF-α) production, whereas IL-11, another member of the family, caused a decrease in TNF-α production (Cork *et al., 2001).*

In conclusion, this study shows that hCG treatment of human EEC in culture increases LIF secretion and inhibits IL-6 secretion in a concentration-dependent manner. Other growth factors present at the embryonic-endometrial interface (IGFs and TGF-βl) also promote endometrial secretion of the pro-implantatory LIF, while TGF-β1 strongly inhibits endometrial production of the pro-inflammatory IL-6. These observations suggest that the human blastocyst may contribute both to its implantation through an increase of endometrial LIF secretion and to its tolerance through an inhibition of endometrial IL-6 production.

Acknowledgements

Sophie Perrier d'Hauterive is Research Assistant and V. Geenen is Research Director at the National Fund of Scientific Research (NFSR) of Belgium. These studies were supported by the Fondation Léon Fredericq (Liège Medical School), NFSR and by the Fédération Belge contre le Cancer.

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