Endometrial interleukin-6 in vitro is not regulated directly by female steroid hormones, but by pro-inflammatory cytokines and hypoxia

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Endometrial interleukin-6 (IL-6) mRNA has been reported to be suppressed in the mid-secretory phase in patients with recurrent early spontaneous abortions. This prompted our study concerning the regulation of endometrial IL-6 in cell culture models of endometrial epithelial and stromal cells. Steroid-dependent secretion of IL-6 was analysed by 17β-estradiol (10^-8 mol/l) or progesterone (10^-6 mol/l) treatment and withdrawal (n = 8). Regulation by pro-inflammatory cytokines was studied in co-cultures of endometrial cells with human blood peripheral mononuclear cells (PBMC; n = 5) and by stimulation with IL-1β, IL-6 and tumour necrosis factor α (TNFα), secreted by PBMCs at high concentrations. Regulation by hypoxia was assessed by culture of endometrial cells in 2% oxygen for 6 and 24 h (n = 5). IL-6 mRNA and protein levels were analysed by RT-PCR and enzyme-linked immunosorbent assays respectively. Endometrial IL-6 was not directly affected by 17β-estradiol and/or progesterone. Co-culturing endometrial cells with PBMCs led to an increase of stromal but not epithelial IL-6 mRNA levels. In stromal cells, IL-6 secretion increased 2–10-fold if stimulated with 10 ng/ml recombinant IL-1β or TNFα (P < 0.05). Hypoxia stimulated IL-6 secretion in epithelial cells up to 2-fold and in stromal cells up to 48-fold (P < 0.05). In conclusion, IL-6 expression in stromal and epithelial cells in vitro is regulated differently by pro-inflammatory cytokines and hypoxia. These results suggest a tight and specific network of control for this important cytokine within different endometrial compartments.

Key words: cytokines/endometrium/hypoxia/interleukin-6/steroids

Introduction

Interleukin-6 (IL-6) is a pleiotropic cytokine which is produced by different types of immune and non-immune cells (Tovey et al., 1988). In human endometrium it is expressed at maximum concentrations in the mid- to late secretory phases (Tabibzadeh et al., 1995), indicating a role in the regulation of endometrial function and in implantation. Recent findings support a role of IL-6 in the establishment of pregnancy because endometrial mRNA is suppressed in the mid-secretory phase of patients with recurrent abortions (Lim et al., 2000; von Wolff et al., 2000). These findings prompted our interest in the regulation of endometrial IL-6 expression. Maximal concentrations of endometrial IL-6 in mid- and late secretory phases (Tabibzadeh et al., 1995; von Wolff et al., 2002) suggest regulation by female steroid hormones, hypoxia or cytokines known to be maximally expressed in the secretory phase.

Previous studies on the regulation of endometrial IL-6 by estrogen and progesterone have not provided uniform results. IL-6 secretion was stimulated in endometrial epithelial cells by progesterone and the combination of 17β-estradiol and progesterone (Laird et al., 1993). However, down-regulation of IL-6 by both steroid hormones was reported in cells from the late secretory phase (Laird et al., 1993). In stromal cells there is evidence that IL-6 is up-regulated by 17β-estradiol (Tseng et al., 1996) but down-regulated by 17β-estradiol if induced by IL-1α (Tabibzadeh et al., 1989) or if taken from the late secretory phase (Laird et al., 1993). These somewhat conflicting results were expanded by studies of Lim et al. (Lim et al., 2000), and our group (von Wolff et al., 2000). Although endometrial IL-6 expression was suppressed in patients with early recurrent abortions, blood levels of estradiol and progesterone were normal. These findings suggest that endometrial IL-6 is not solely regulated by female steroid hormones.

Previous studies support a role for pro-inflammatory cytokines in the regulation of endometrial IL-6. IL-1, a cytokine that seems to play an important role in murine implantation (Simon et al., 1998), shows an endometrial expression pattern similar to IL-6 (von Wolff et al., 2000). IL-1 stimulates IL-6 expression in a dose-dependent fashion (Laird et al., 1994; Vandermolen and Gu, 1996). Expression of both cytokines is simultaneously suppressed in the mid-secretory phase of patients with recurrent early abortions (von Wolff et al., 2000).

Recent studies on the regulation of endometrial vascular endothelial growth factor (VEGF) (Popovic et al., 1999; Sharkey et al., 2000) have shed new light on endometrial hypoxia as a factor possibly affecting the regulation of endometrial function. As IL-6 has been
shown to be stimulated by hypoxia in different cell types such as placental villous explants, immune cells and endothelial cells (Benyo et al., 1997; Naldini et al., 1997; Ali et al., 1999), we investigated whether hypoxia affects endometrial IL-6 production.

To study the regulation of endometrial IL-6 production we closely characterized and optimized our cell culture models. These models were used to examine effects of 17β-estradiol, progesterone and hypoxia. Regulation by pro-inflammatory cytokines was studied in co-culture models in which epithelial and stromal cells were initially stimulated by a spectrum of cytokines, secreted by co-cultured mononuclear blood cells. Additionally, cells were stimulated with individual recombinant cytokines at concentrations in the range of those found in the co-cultures.

Materials and methods

Cell culture

Isolation and culture of endometrial tissue was performed according to Classen-Linke et al. (Classen-Linke et al., 1997). In brief, endometrium was taken from premenopausal patients undergoing hysterectomy for benign reasons. Informed consent was obtained from each woman participating in the study and ethical approval was obtained from the institutional ethical board of the University of Heidelberg. Separation of stromal and epithelial cells was performed by filtration of minced endometrium through a 180 μm nylon membrane (Millipore, Eschborn, Germany) followed by a 40 μm nylon sieve (Becton Dickinson, NJ, USA). Epithelial glands were trapped on the second sieve and seeded at a density of 6000 endometrial glands/cm² on 12 mm-diameter filters (pore size 0.4 μm) in Millicell CM (Millipore) inserts, previously coated with 0.1 ml of 1:4 diluted matrigel without phenol red (Becton Dickinson). Inlets were then placed in 24-well tissue culture plates (Falcon, Oxnard, USA) and incubated in DMEM/F-12HAM medium without phenol red (Sigma) containing antibodies, anticytokines and fetal calf serum (FCS) (Gibco, Eggengstein, Germany) at 37°C (5% CO₂) until 50–90% confluence was reached.

Stromal cells that passed the 40 μm sieve, were thoroughly washed and purified by extraction of immune and endothelial cells using CD4+, CD56-, CD45- and CD31-positive magnetic Dynabeads (Dynal, Oslo, Norway). To further reduce contamination of cell cultures by non-stromal cells, cells were passed once: cells were cultured for 2 days, released by incubation with 10% trypsin (Gibco) and seeded in 24-well tissue culture plates (Falcon) with a density of 100 000 cells/well.

Cells were cultured for 4–5 days, followed by stimulation with 17β-estradiol (10⁻⁸ mol/l = 2.7 ng/ml) and/or progesterone (10⁻⁶ mol/l = 310 ng/ml) (n = 8) or with IL-1ß, IL-6 or tumour necrosis factor α (TNFα) (0.1 ng/ml, 10 ng/ml) for 6 and 24 h (n = 4). After 6 or 24 h, supernatants were snap frozen in liquid nitrogen for enzyme-linked immunosorbent assay (ELISA) studies. Negative controls were cell cultures without stimulation by 17β-estradiol and/or progesterone.

To analyse the effect of estrogen and progesterone withdrawal on endometrial IL-6 secretion, cells were cultured for 4–5 days under stimulation with 17β-estradiol and progesterone as described above. One or both hormones were then completely withdrawn by washing the cells with PBS three times and incubating them in media without hormones or hormone-like ingredients for 6 or 24 h. Supernatants were then snap frozen for ELISA studies (n = 6).

The effect of low oxygen on epithelial and stromal IL-6 secretion was studied in a separate incubator, injected with N₂ to maintain an O₂ concentration of 2% (1.5–2.5%) (5% CO₂). Cells were cultured as described above for 6 or 24 h under hypoxic or normoxic (controls, 21% O₂) conditions. Cells and culture supernatants were snap frozen for ELISA studies and semi-quantitative RT–PCR. Hypoxia-induced endometrial VEGF production (Popovici et al., 1999; Sharkey et al., 2000) was used to validate the culture system. All experiments were performed in duplicates.

Co-culture of epithelial and stromal cells with blood peripheral mononuclear cells (PBMCs)

PBMCs were isolated from the buffy coat from healthy blood donors by Ficoll-Hypaque density gradient centrifugation (Vacutainer CPT Cell Preparation Tube; Becton Dickinson, Franklin Lakes, USA). To perform all experiments with PBMCs from one individual donor, PBMCs were cryopreserved in RPMI-1640 medium without phenol red, inactivated FCS (40%) and dimethyl sulfoxide (DMSO) (15%) (all from Sigma, Deisenhofen, Germany) in liquid nitrogen. PBMCs were seeded at a density of 50 000 or 500 000 per well into Millicell CM inserts (Millipore) and 24-well tissue culture plates (Falcon). Cells were first cultured in RPMI-1640 containing 100 ng/ml lipopolysaccharide (LPS) (Sigma) for 5 days to initiate differentiation of monocytes to macrophages. Characterization of different cell types was performed after 5 days of culture (n = 3) in three cultures by flow cytometry analysis using antibodies directed against T lymphocytes (CD4, CD8; Dako, Glostrup, Denmark), B lymphocytes (CD19; Diaclone Research, Besancon, France), monocytes/macrophages (CD14; Diacclone), and natural killer cells (CD16+CD56; Immunotec, Marseille, France). Cell viability was determined by day 5 as described below. IL-1ß, IL-6, leukemia inhibitory factor (LIF) and TNFα secretion of PBMCs was measured by ELISA after 5 days of culture (n = 3). On day 5, inerts or wells containing PBMCs were added to confluent epithelial cells (in inlets) or stromal cells (in wells) and cultured in DMEM/F-12HAM medium without phenol red (Sigma) for 24 h. Epithelial and stromal cells were transferred into vials containing guanidine isothiocyanate (GTC) solution and analysed for IL-6 mRNA by semi-quantitative RT–PCR.

Characterization of cell cultures

To characterize the composition of epithelial and stromal cell cultures, four stromal and epithelial cell cultures were investigated by immunocytochemistry after 5–6 days by use of monoclonal antibodies. Anti-leukocytes (CD45; 1:100; Dako), anti-monocytes/macrophages (CD14; 1:20; Dako), anti-natural killer cells (CD31; 1:50; Dako) were diluted as recommended by the suppliers. For immunocytochemistry, commercially available kits were used (Histostain-SP Kits; Zymed Laboratories, San Francisco, CA, USA).

Cell viability of cultured stromal and epithelial cells was analysed in four cultures after 6 and 12 days of cell culture by use of a LIVE/DEAD Viability/Cytotoxicity Kit (Mo Bi Tec, Göttingen, Germany). Live cells were distinguished by green fluorescence and dead cells were distinguished by red fluorescence.

To determine the effect of several passages on stromal cell function, we analysed the potential of stromal cells to decidualize after one, three, six and nine passages (n = 3). Cells were cultured as described above, and passaged once a week. Decidualization was achieved by stimulating 100 000 stromal cells per well with 17β-estradiol (0.5 ng/ml) and progesterone (50 ng/ml) for 20 days. Decidualization was confirmed by high levels of prolactin and insulin-like growth factor binding protein 1 (IGFBP-1) in the supernatant.

ELISA

Concentrations of IL-1ß, IL-6, LIF, TNFα and IFGFP-1 in culture supernatants were determined using commercially available ELISA kits (IL-1ß, IL-6, TNFα: Laboserv Diagnostica, Giessen, Germany; LIF: R&D-Systems, Minneapolis, MN, USA; IGFBP-1: Diagnostics Systems Laboratories, Webster, TX, USA). The detection limits of the ELISAs were 3.9 pg/ml (IL-1ß), 0.2 pg/ml (IL-6), 15 pg/ml (TNFα), 0.8 pg/ml (LIF) and 0.25 ng/ml (IGFBP-1). The intra- and inter-assay variations of the ELISAs were 4.1 and 6.5% (IL-6), 15 pg/ml (TNFα), 2.7 ng/ml (LIF) and 0.25 ng/ml (IGFBP-1). The ELISAs were performed according to the instructions of the manufacturer. Prolactin was measured by use of a commercial electrochemiluminescence immunoassay (Elecys Prolactin Immunoassay; Roche Diagnostics, Mannheim, Germany).

RT–PCR

Cells were washed (epithelial cells) or scraped (stromal cells) out of the culture wells, transferred into a solution containing GTC and stored at –70°C. RNA isolation and DNA digestion was performed with High Pure RNA Isolation Kits (Roche Diagnostics). First-strand cDNA was synthesized using First Strand Synthesis Kits (Roche Diagnostics), followed by PCR (PCR Core Kit; Roche Diagnostics). The housekeeping gene cytochrome C oxidase subunit 1 (COI) was co-amplified in a companion tube. Two sets of IL-6 primers were designed across intron-exon boundaries and yielded a 327-bp product: sense (5’-CTCTCTCCACAACGCGCTCT-3’) and antisense (5’-GGCGGACTCTTCCTCATTCAATC-3’) or a 628-bp product: sense
(5′-ATGAACTCTCTCCAAGCGC-3′) and antisense (5′-GAAGAGCCCTAGGCTGAGCT-3′). The COI primers yielded a 268-bp product: sense (5′-CGTCACAGCCCATGACTT-3′) and antisense (5′-GTT TAGGCTCACGGGACCT-3′). The numbers of cycles were within the linear logarithmic phase of the amplification curve. Thirty-five cycles were used for IL-6 and 25 cycles for COI and amplified as follows: 1 min at 92°C, 1 min at 54°C (IL-6) or 51°C (COI) respectively and 1 min at 72°C. Identity of the PCR products was confirmed by sequencing. The PCR products were separated electrophoretically in a 1% agarose gel. Semi-quantification of mRNA levels was achieved by normalizing the optical densities of the specific bands to the optical densities of the housekeeping gene COI.

Statistical analysis
Wilcoxon ranked sign test was used for statistical evaluation. Significance was assumed at the P < 0.05 level.

Results
Characterization of cell cultures
Immunocytochemistry of epithelial and stromal cell cultures (n = 4) revealed contamination of cultures with 0–5% immune cells and 0–2% endothelial cells. As purification of cells by magnetic beads further decreased contamination by 50%, cells were additionally purified by magnetic beads before seeding.

Cell viability of epithelial and stromal cells was assessed on days 6 and 12 of cell culture. Viability was 94–98% on day 6 and 91–97% on day 12, indicating adequate viability of epithelial and stromal cells at the time experiments were performed (days 4–5).

To decrease contamination of stromal cell cultures, it is generally recommended to passage cell cultures several times. To study cell function after several cell passages, we analysed the potential of stromal cells to decidualize after one, three, six and nine passages. After one passage, stromal cells had decidualized well, as shown by secretion of high levels of prolactin (114 ng/ml) and IGFBP-1 (980 ng/ml). Decidualization was reduced after three and more passages, indicating impaired cell function (prolactin, 54 ng/ml; IGFBP-1, 11 ng/ml). Consequently, stromal cells were only passaged once.

17β-estradiol and progesterone stimulation and withdrawal
Endometrial epithelial and stromal cells were first stimulated with 17β-estradiol and/or progesterone without prior extraction of immune and endothelial cells by magnetic beads. IL-6 concentrations as measured by ELISA revealed a wide spectrum of IL-6 levels with great inter-assay variations. Introduction of magnetic beads to purify cell cultures resulted in reduced variations of IL-6 concentrations and reduction of basal IL-6 levels by ~50%, indicating reduction of contaminating IL-6-producing immune cells.

Untreated epithelial control cultures secreted on average 1260 pg/ml IL-6 (±550). Untreated stromal cells secreted 86 pg/ml (±45). Treatment with 17β-estradiol, progesterone or the combination of both steroids for 24 h revealed no significant effect on endometrial IL-6 secretion (Figure 1).

Withdrawal of 17β-estradiol, progesterone or the combination of both hormones for 24 h also had no significant effect on IL-6 concentrations in culture supernatants (Figure 1). These results were independent of the concentration of 17β-estradiol and progesterone. Preliminary studies with 10 cell cultures stimulated with a 10 times lower concentration of 17β-estradiol (10−5 mol/l = 0.27 ng/ml) and/or progesterone (10−5 mol/l = 31 ng/ml) also had no significant effect (data not shown).

Endometrial tissues for cell cultures were taken at different phases of the menstrual cycle (cycle days 12, 13, 14, 19, 20, 21, 22). No systematic correlation of IL-6 secretion with the menstrual phase as determined by histological evaluation and progesterone levels was observed.

The lack of effect of 17β-estradiol and/or progesterone on IL-6 was confirmed by IL-6 mRNA analysis by RNase protection assay (von Wolff et al., 2002) after 6 h of stimulation or withdrawal (data not shown).

Cytokine-dependent expression of IL-6: stimulation with cytokines secreted by PBMCs
Endometrial epithelial and stromal cells were co-cultured with PBMCs to expose cells to a spectrum of pro-inflammatory cytokines. PBMCs were first treated with LPS for 5 days prior to co-culture. Three representative PBMC cultures were characterized by flow cytometric analysis and revealed on average 20% monocytes/macrophages, 10% CD4-positive T lymphocytes, 25% CD8-positive T lymphocytes, 5% CD19-positive B lymphocytes and 5% CD16+CD56-positive natural killer cells. Forty percent of cells were negative with the phenotypic markers used. 500 000 PBMCs secreted IL-1β (271–321 pg/ml). IL-6 (48–57 pg/ml) and TNFα (211–261 pg/ml). These cytokines were also used individually for stimulation of endometrial cell cultures (see below). LIF concentrations were below the detection limit (8 pg/ml). IL-6 mRNA concentrations were analysed by RT–PCR after 24 h of co-culturing PBMCs with endometrial epithelial and stromal cells. In epithelial cells, IL-6 mRNA concentrations were not influenced by co-culturing cells with PBMCs (Figure 2). In contrast, stromal cells revealed increasing IL-6 mRNA concentrations in co-cultures. The increase in IL-6 mRNA concentrations was greater with a higher number of co-cultured PBMCs (Figure 2).

Cytokine-dependent expression of IL-6: stimulation with IL-1β, IL-6 and TNFα
Endometrial epithelial and stromal cells were stimulated with recombinant IL-1β, IL-6 and TNFα as these had been secreted at high concentrations by co-cultured PBMCs. In cell cultures stimulated by IL-1β and TNFα, IL-6 concentrations were analysed by ELISA. In cell cultures challenged with IL-6 itself, IL-6 levels could not be analysed by ELISA due to high concentrations of recombinant IL-6 in the culture supernatants. IL-6 analysis had therefore to be performed by RT–PCR.

Corresponding to our co-culture experiments, incubation with IL-1β, TNFα (Figure 3) or IL-6 (data not shown) did not stimulate IL-6 expression in epithelial cells. In contrast, IL-6 expression in stromal cells was stimulated by IL-1β and TNFα (Figure 3) but not by IL-6 (data not shown). Stimulation by IL-1β and TNFα resulted in significant (P < 0.05) and dose-dependent (2–10-fold) increases of IL-6 concentrations.

Hypoxia and stimulation of IL-6
In epithelial cell cultures, hypoxia led to an increase of IL-6 secretion in four out of five cell cultures (Figure 4). The increase in IL-6 was maximally 2-fold after 24 h. In contrast, hypoxia-induced IL-6 secretion in stromal cells was much higher. IL-6 secretion in stromal cells increased significantly (4–48-fold) after 24 h exposure to hypoxia (Figure 4).

IL-6 stimulation by hypoxia was confirmed by semi-quantitative RT–PCR as shown by representative cell cultures (Figure 5). IL-6 mRNA expression increased in four out of five epithelial cell cultures when stimulated by hypoxia. In stromal cells, IL-6 mRNA stimulation was found in all cell cultures. Stimulation of VEGF mRNA expression by hypoxia was used as positive control. VEGF concentrations in the supernatants, as measured by ELISA, were also increased 2.4-fold (±0.6) in epithelial cells and in stromal cells 5.2-fold (±2.2) (data not shown). Stimulation of IL-6 secretion by hypoxia could not be
Regulation of endometrial IL-6

Figure 1. Interleukin-6 (IL-6) concentrations in the supernatants of epithelial cell cultures (grey boxes) and stromal cell cultures (white boxes) after treatment with 17β-estradiol (E₂) (10⁻⁸ mol/l = 2.7 ng/ml) or progesterone (P) (10⁻⁶ mol/l = 310 ng/ml) (n = 8) for 6 and 24 h and after 17β-estradiol or progesterone withdrawal. Untreated epithelial control cultures secreted on average 1260 pg/ml IL-6 (±550). Untreated stromal cells secreted much less IL-6 (86 pg/ml, ±45).

Figure 2. Interleukin-6 (IL-6) mRNA expression in epithelial and stromal cells, co-cultured with peripheral blood mononuclear cells (PBMCs). Endometrial cells were co-cultured with 50 000 and 500 000 PBMCs/well for 24 h. PBMCs were isolated from peripheral blood by density gradient centrifugation and stimulated with 100 ng/ml lipopolysaccharide for 5 days and found to secrete high concentrations of cytokines such as IL-1β, IL-6 and tumour necrosis factor (TNF) α. IL-6 mRNA-expression was analysed by semi-quantitative RT–PCR in five different cell cultures. Co-amplification of the housekeeping gene cytochrome oxidase subunit I (COI) revealed equal loading with cDNA and constant PCR conditions.

correlated with the menstrual phase at which the tissue samples were collected (data not shown).

Discussion

Endometrial IL-6 is expressed at high concentrations in the mid- to late secretory phase of the menstrual cycle (Tabibzadeh et al., 1995; von Wolff et al., 2002). High concentrations in the mid-secretory phase suggest regulation of IL-6 expression by 17β-estradiol and progesterone stimulation whilst high concentrations in the late secretory phase might suggest regulation by 17β-estradiol and progesterone withdrawal.

Previous studies with cultured endometrial cells have already analysed endometrial IL-6 regulation by 17β-estradiol and progesterone and have reported conflicting results. Both stimulation and suppression of endometrial IL-6 secretion by 17β-estradiol and progesterone have been described (Tabibzadeh et al., 1989; Laird et al., 1993; Tseng et al., 1996). Other studies involving the regulation of IL-6 in non-endometrial cells have not helped to clarify these conflicting results: phorbol ester-induced activation of the IL-6 promoter in an endometrial adenocarcinoma cell line was inhibited by 17β-estradiol (Ray et al., 1997) and 17β-estradiol inhibits IL-6 secretion in stromal cells from other tissues such as murine bone stromal cells (Girasole et al., 1992).

It is a matter of debate, whether the different results are due to the heterogeneity of tissue samples collected from hysterectomy specimens or to different culture conditions. Tabibzadeh et al. (Tabibzadeh et al., 1989) performed stimulation experiments with stromal cells, passaged the cells up to four times and used medium without FCS. Laird et al. (Laird et al., 1993) cultured epithelial and stromal cells in plastic wells with medium containing serum without further passages. Vandermolen and Gu (Vandermolen and Gu, 1996) used tissue aggregates instead of separated epithelial and stromal cells and Tseng et al. (Tseng et al., 1996) cultured unpassaged stromal cells in medium containing 2.5% FCS. These different culture conditions might influence cell culture experiments. As cell culture
Figure 3. Interleukin-6 (IL-6) concentrations in culture supernatants of four different epithelial and stromal cell cultures after stimulation with IL-1β or tumour necrosis factor (TNF) α for 24 h. IL-1β or TNFα concentrations were similar to those secreted by peripheral blood mononuclear cells in co-cultures (Figure 2). IL-6 concentrations in culture supernatants were analysed by ELISA.

Figure 4. Interleukin-6 (IL-6) concentrations in supernatants of endometrial cells, cultured under hypoxic conditions. Five different semi-confluent epithelial and stromal cell cultures were incubated in duplicates for 6 or 24 h under hypoxia [O_2, 2% (1.5–2.5%); CO_2, 5%]. IL-6-expression was analysed in the supernatants by ELISA and compared with normoxic controls.

on plastic surfaces can influence cell function (Mullholand et al., 1988; Schatz et al., 1990; White et al., 1990), we cultured cells on Matrigel in dual-chambered systems in which cells maintain cell polarity (Schatz et al., 1990; White et al., 1990; Classen-Linke et al., 1997) and express both estradiol and progesterone receptors (Classen-Linke et al., 1997). Furthermore, as several passages of stromal cells may change cell function and as cell culture in serum-free medium induces apoptosis in endometrial epithelial cells (M.von Wolff, unpublished data), stromal cells were passaged once and cultured in medium with 1% FCS.

After carefully excluding adverse culture conditions, we did not find any direct effect of 17β-estradiol and/or progesterone treatment or withdrawal on endometrial IL-6 secretion. However, IL-6 is expressed at maximum levels in endometrial epithelial cells in the mid- to late secretory phase, the time when endometrium is exposed to maximum progesterone and estradiol blood concentrations. It can
therefore be speculated that stromal and epithelial IL-6 is not directly regulated by 17β-estradiol and progesterone, but indirectly by the interaction with other steroid-dependent cells, by other mediators such as pro-inflammatory cytokines which are expressed at maximum concentrations in the late secretory phase (von Wolff et al., 2000) or by tissue hypoxia.

The relevance of the interaction of other steroid-dependent cells in the regulation of epithelial or stromal function has been shown by Cooke et al. (Cooke et al., 1998) and others. Cooke et al. demonstrated in transgenic mice that estrogen stimulates mitogenesis in endometrial epithelial cells indirectly by stimulation of estrogen-dependent stromal cells. However, our experimental design did not allow us to draw conclusions concerning the indirect stimulation of epithelial and stromal cells by steroids.

To analyse the regulation of IL-6 by other mediators such as pro-inflammatory cytokines, we set up a co-culture model to stimulate endometrial cells with a spectrum of those cytokines released from mononuclear blood cells. This model was chosen as the relevance of direct stimulation of endometrial cells by arbitrary concentrations of recombinant cytokines is difficult to interpret. PBMCs produce cytokines and can be found at high concentrations in the endometrium of mid- and late secretory phases (von Wolff et al., 2000). Furthermore, the availability of these cells allowed us to set up reproducible culture conditions.

Previous studies have reported stimulation of IL-6 secretion in endometrial epithelial cells (Laird et al., 1994) and in endometrial biopsy specimens by inflammatory cytokines (Vandermolen and Gü, 1996). Stimulation of IL-6 expression in stromal cells by IL-1β and TNFα (Tabibzadeh et al., 1989) was confirmed in our study. In contrast to Laird et al. (Laird et al., 1994), we did not find stimulation of IL-6 secretion in epithelial cells, neither in our co-cultures nor by direct stimulation with recombinant cytokines. Disturbance of our results by contaminating immune cells could almost be excluded as stimulation of IL-6 secretion was found only in stromal cells and not in epithelial cell cultures.

Hyoxia is a well known stimulatory factor of IL-6 production in different cell types such as PBMCs (Naldini et al., 1997) and endothelial cells (Yan et al., 1997). IL-6 release is stimulated by hyoxia-induced activation of the nuclear factor-IL-6 (NF-IL-6) site (Yan et al., 1997) and activation of NF-kappa B (Koong et al., 1994). Our IL-6 production is not only stimulated in mononuclear blood cells and endothelial cells, which are found at high concentrations in endometrium, but, as our results show, also in human endometrial stromal cells and to some degree in epithelial cells. Previous studies support our results indicating that hyoxia is involved in the regulation of endometrial stromal and epithelial cells. Incubation of endometrial stromal and epithelial cells (Sharkey et al., 2000) and of decidualized stromal cells (Popovici et al., 1999) in hyoxia (2% O2) leads to a significant increase of VEGF production.

Maximum IL-6 expression in the mid- and late secretory phases in epithelial cells suggest that IL-6 might play a role in implantation (Tabibzadeh et al., 1995). IL-6 is secreted into the uterine cavity (von Wolff et al., 2002) where it might act on the implanting blastocyst. Previous studies have suggested that endometrial overexpression and underexpression of IL-6 may affect fertility. Elevated endometrial IL-6 concentrations have been found in women suffering from endometriosis and unexplained infertility (Tseng et al., 1996) and in patients with intrauterine contraceptive devices (Ammälä et al., 1995). In contrast, suppression of endometrial IL-6 has been described in patients with recurrent abortion (Lim et al., 2000; von Wolff et al., 2000).

Our results indicate that endometrial IL-6 is not directly regulated by 17β-estradiol and progesterone in vitro. It is however regulated by pro-inflammatory cytokines such as IL-1β and TNFα and by hyoxia. The late secretory phase and menstruation are characterized by high expression of pro-inflammatory cytokines (von Wolff et al., 2000) and by the induction of hyoxia arising from vasoconstriction of the spiral arteries (Markee, 1940). Progressive increases in IL-6 concentrations during the secretory phase (Tabibzadeh et al., 1995; von Wolff et al., 2000) therefore not only suggests a role for IL-6 in the regulation of the endometrium around the implantation window during the mid-secretory phase, but also some function in menstrual shedding in the late secretory phase. Menstrual shedding shows many parallels with the inflammation process, characterized by production of pro-inflammatory cytokines such as IL-1 and TNFα, by tissue oedema and by hyoxia. High levels of pro-inflammatory cytokines such as IL-1β and TNFα, not only stimulate IL-6 expression in endometrial stromal cells but also affect the activity of endometrial

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**Figure 5.** Interleukin-6 (IL-6) mRNA-expression in representative epithelial and stromal cell cultures, cultured under hyoxia conditions as described in Figure 4. IL-6 mRNA expression was analysed in epithelial and stromal cells by semi-quantitative RT-PCR and compared to normoxic controls. Co-amplification of the housekeeping gene cytochrome oxidase subunit I (COI) revealed equal loading with c-DNA and constant PCR conditions.
immune cells. TNFα has been associated with menstrual shedding as a factor inducing apoptosis (Tabibzadeh, 1994) and tissue oedema by damaging endothelial cells (Tabibzadeh, 1996). As IL-6 and TNFα expression are tightly regulated and IL-6 secretion is stimulated by TNFα, it can be speculated that high levels of IL-6 contribute directly or through other cytokines to the process of menstrual shedding.

In summary, IL-6 is not directly regulated by 17β-estradiol or progesterone in endometrial epithelial and stromal cells in vitro. IL-6, however, is strongly stimulated in stromal cells by hypoxia, IL-6 in the regulation of endometrial function.

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