

The production of leukaemia inhibitory factor by human endometrium: presence in uterine flushings and production by cells in culture

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The concentration of leukaemia inhibitory factor (LIF) was measured in uterine flushings obtained from normal fertile women, from women with unexplained infertility and from women who suffered recurrent miscarriage. In normal fertile women, LIF was not detected in flushings obtained on days luteinizing hormone (LH)+0 to LH+6 of the cycle, but concentrations gradually increased from day LH+7 to a maximum at day LH+12. The amount of LIF in flushings obtained from women with unexplained infertility was significantly lower than in those from normal fertile women on day LH+10 ($P < 0.05$). The production of LIF by cultured human epithelial and stromal cells was also investigated. LIF was not detectable in the supernatants of cultured stromal cells. Basal LIF production by epithelial cells varied according to the stage in the cycle at which the biopsy was taken. Significantly more LIF was produced by epithelial cells from late proliferative and early secretory endometrium compared with amounts produced by cells from early proliferative ($P < 0.001$) and late secretory ($P < 0.01$) endometrium. High doses of progesterone and oestradiol caused a small decrease in epithelial cell LIF production: the combined effect of progesterone and oestradiol ($P < 0.01$) was greater than the effect of either steroid alone ($P < 0.05$). The results show, for the first time, the capability of human endometrium to produce LIF *in vivo*. The fact that maximum LIF concentrations are present at implantation and that decreased concentrations occur in women with unexplained infertility suggest the importance of this cytokine in embryo implantation.

Key words: human endometrium/leukaemia inhibitory factor/steroids

Introduction

The production of numerous growth factors and cytokines by human endometrium is well documented (Guidice, 1994), and evidence suggests that these growth factors and cytokines may be important mediators of embryonic–endometrial interactions and thus of the control of embryo implantation (Guidice,

1994). Leukaemia inhibitory factor (LIF) is a secreted glycoprotein which was first described as a factor that induced the differentiation of mouse myeloid leukaemic M1 cells into macrophages (Gearing *et al.*, 1987). Since then, it has been shown to display multiple biological activities including suppression of the differentiation of normal embryonic stem cells (Smith *et al.*, 1988) and stimulation of calcium release from bone (Abe *et al.*, 1986). Recently, the production of LIF by mouse endometrium has been described (Bhatt *et al.*, 1991), and several studies have suggested its importance in the control of implantation in the mouse. In particular, large amounts of LIF mRNA have been found in the glandular epithelium of mouse uterus at implantation (day 4 of pregnancy) (Bhatt *et al.*, 1991). Furthermore, it has also been shown that in female transgenic mice homozygous for LIF gene deficiency, embryo implantation did not occur (Stewart *et al.*, 1992). This lack of implantation was not due to defects in the embryo because implantation of the same embryos did occur when the embryos were transferred to pseudo-pregnant wild-type mothers. Expression of mouse endometrial LIF has also been shown to be under maternal control, and the coincidental expression of LIF in the endometrium with elevated concentrations of oestrogen suggests that it may be controlled by steroid hormones (Stewart, 1994).

The expression of LIF mRNA and protein by human endometrium has also been described recently (Charnock-Jones *et al.*, 1994; Kojima *et al.*, 1994; Chen *et al.*, 1995). However, there are no reports showing the secretion of LIF by human endometrium *in vivo*, and its importance in the control of human embryo implantation has not been investigated. The purpose of this study was to investigate the secretion of LIF by human endometrium *in vivo* by measuring concentrations in uterine flushings obtained from normal fertile women throughout the luteal phase of the menstrual cycle. Its importance in human embryo implantation was determined by comparing concentrations in flushings from normal women with those in the flushings from women with unexplained infertility and from women who suffer recurrent miscarriage. In addition, epithelial cell cultures prepared from endometrial biopsy tissue obtained at different times in the cycle were used to investigate the effects of oestradiol and progesterone on LIF production by these cells.

Materials and methods

Human subjects

Uterine flushings and endometrial biopsy samples were obtained throughout the luteal phase from 21 normal fertile women attending the Jessop Hospital for Women in Sheffield, UK, for sterilization or

hysterectomy. All the women were aged between 24 and 40 years and had regular cycles of between 25 and 35 days. None of the women had taken any steroid hormones for 2 months prior to the study. The number of samples obtained from each woman ranged from one to six, with a minimum interval of 48 h between any two flushings. In all, 44 samples were obtained. In addition, uterine flushings were obtained from women who suffered unexplained infertility or recurrent (three or more) miscarriage recruited from the outpatient clinic at the same hospital. Unexplained infertility was defined as patients who had experienced >18 months of infertility with regular menstrual cycles of between 25 and 35 days, endocrinological evidence of ovulation, normal thyroid function tests and plasma prolactin concentrations, a normal hysterosalpingogram, a normal laparoscopy and a normal semen analysis for their husband. The recurrent miscarriage women were aged between 25 and 40 years and had experienced regular cycles of between 25 and 35 days. For each of these subjects a uterine flushing was obtained on days luteinizing hormone (LH)+7, LH+10 or LH+12 of the cycle. A total of 22 samples were obtained from women with unexplained infertility and 33 samples from women who suffered recurrent miscarriage. A single flushing was obtained from each woman. The flushings obtained from all women were precisely timed according to the LH surge, with the day of the surge being day LH+0. Local ethical committee approval was given for the collection of flushings, and informed consent was obtained from all participating subjects.

LH assay

Starting on day 9 of the cycle, each subject collected daily plasma or early morning urine specimens for LH assay. The LH in these samples was measured by an immunoradiometric assay as described previously (Li *et al.*, 1993a).

Uterine flushing

This was performed as an outpatient procedure as described previously (Li *et al.*, 1993a). A bivalve speculum was inserted, through which a size 8 Foley catheter was introduced into the uterine cavity and the balloon of the catheter inflated with 1 ml normal saline. Following this, 2 ml saline solution were gradually flushed into the uterine cavity via the opening connected to the inner lumen; afterwards, gentle suction via the same opening was applied to recover the fluid. The procedure was repeated five times, each time using 2 ml fresh saline solution. A total of 10 ml saline were used to carry out the flushing. The samples were pooled, aliquoted into 1 ml fractions and stored at -20°C for the LIF assay.

Cell culture

Endometrial tissue for culture was obtained from subjects undergoing laparoscopic sterilization or hysterectomy: biopsies were obtained from five women undergoing hysterectomy and from 16 women undergoing sterilization. Informed consent was obtained from all women participating in the study. Human endometrial epithelial and stromal cells were prepared and cultured as described previously (Laird *et al.*, 1993). The endometrial biopsy samples were collected in Hanks' balanced salt solution containing streptomycin and penicillin (100 $\mu\text{g}/\text{ml}$). The tissue was chopped finely with scissors and incubated at 37°C for 45 min in 5 ml Dulbecco's modified Eagle's medium (DMEM) containing 0.2% collagenase (type 1a) (DMEMC). During and at the end of the incubation the tissue was pipetted gently to disperse the cells. The epithelial cells were separated from the stromal cells by centrifugation at 100 *g*. The supernatant containing stromal cells was removed, and the pellet which contained mainly epithelial cells present as glands was incubated at 37°C for a further 45 min in 5 ml DMEMC. The cells were dispersed again by gentle pipetting

and the epithelial cells pelleted by centrifugation at 100 *g*. The stromal cells present in the supernatant were pooled with those from the first supernatant and pelleted at 300 *g*.

The epithelial and stromal cells were purified further by unit density sedimentation. Each cell type was resuspended in 2 ml DMEM containing fetal calf serum (10%), glutamine (4 mmol/l), penicillin and streptomycin (100 $\mu\text{g}/\text{ml}$) (CDMEM), and gently pipetted onto 8 ml CDMEM in a test tube and left for 30 min at room temperature. Stromal cells were taken from the top 8 ml and used for cell culture. For the epithelial cells, the top 8 ml were discarded and the cells in the lower 2 ml were used for cell culture. Each cell type was plated onto 96-well plates at $\sim 0.8 \times 10^5$ cells per well. The cells were grown in CDMEM medium to confluency (usually 48–72 h) at 37°C in an atmosphere of 5% CO_2 and 95% air. At confluency, the medium was changed and replaced with CDMEM containing [^3H]thymidine (1 $\mu\text{Ci}/\text{well}$), and either no supplements or oestradiol (10^{-6} M), progesterone (10^{-6} M) or oestradiol and progesterone combined (each at 10^{-6} M). The cells were grown for a further 48 h, after which the supernatants were removed and stored at -20°C for the analysis of LIF. The cells were then washed in PBS, harvested onto filter paper and the radioactivity incorporated in the cells counted using a β -counter. The purity of the cell cultures at the end of the culture period was verified using immunocytochemistry to show the expression of vimentin (stromal cell marker), cytokeratin (epithelial cell marker) and anti-CD45 (leukocyte marker), as described previously (Laird *et al.*, 1993).

LIF assay

An enzyme-linked immunoassay kit specific for human LIF was purchased from Amersham Canada Ltd (Oakville, Ontario, Canada). The assay was performed with the aid of a microplate reader attached to an automatic well-washing apparatus (Beckman Instruments Inc., Fullerton, CA, USA). The supplier's instructions for the assay procedure were modified slightly to increase assay sensitivity. Samples were thawed and 100 μl added to the wells of a 96-well microplate which had been pre-coated with specific anti-human LIF antibody. The plate was incubated for 16–18 h at room temperature instead of 37°C , as suggested by the supplier, to reduce background. Each well was then washed with the washing buffer included in the kit. Secondary antibody conjugated with alkaline phosphatase (ALP) was added to each well and the plate incubated for a further 2 h. Unbound antibody was removed by washing and the phosphatase substrate was added. After incubation for 30 min, the absorbance at 490 nm was read using a plate reader. The assay sensitivity (two times the SD of the blank) was 2 pg. Intra- and interassay variations were 8 and 14% respectively. An analysis of the flushings and cell culture supernatants to which known amounts of LIF had been added confirmed the linearity of the assay for the measurement of LIF in these fluids, and there was no detectable LIF present in the CDMEM in which the cells were grown.

Statistical analysis

The Mann–Whitney (non-parametric) *U*-test was used to assess differences between the concentration of LIF in the flushings from fertile, infertile and recurrent miscarriage women and for LIF production by cells obtained at different times in the cycle and in the presence or absence of steroids.

Results

Figure 1 shows the amounts of LIF found in the uterine flushings obtained from normal fertile women throughout the luteal phase of the menstrual cycle. The concentrations of LIF in flushings obtained on days LH+0 to LH+6 were below the

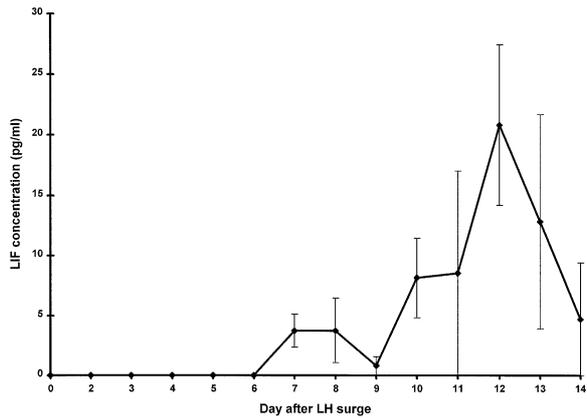


Figure 1. The concentration of leukaemia inhibitory factor (LIF) in uterine flushings obtained from normal fertile women throughout the secretory phase of the menstrual cycle. Day 0 is the day of the luteinizing hormone (LH) surge. Values shown are means \pm SEM ($n = 1, 2, 1, 4, 1, 2, 4, 5, 2, 7, 2, 8, 3$ and 2 for days LH+0, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 and 14 respectively). The detection limit for the assay was 2 pg/ml.

sensitivity of the assay. After day LH+7, LIF concentrations gradually increased through the remainder of the cycle, and reached a maximum at day LH+12.

Figure 2 compares the amounts of LIF in the uterine flushings obtained on days LH+7, LH+10 and LH+12 of the menstrual cycle from normal fertile women, women with unexplained infertility and women who suffer recurrent miscarriage. A greater proportion of infertile women had undetectable levels of LIF in their flushings on each of the days studied compared with normal fertile women and women who suffer recurrent miscarriage: for day LH+7 these were 87.5 (infertile), 25 (fertile) and 29% (recurrent miscarriage); for day LH+10 these were 100 (infertile), 43 (fertile) and 36% (recurrent miscarriage); and for day LH+12 these were 57 (infertile), 12.5 (fertile) and 50% (recurrent miscarriage). Concentrations of LIF in the flushings obtained from infertile women on day LH+10 were significantly less than those in the flushings obtained from fertile women on the same day ($P < 0.05$). There was no significant difference in the amounts of LIF in the flushings from women who suffer recurrent miscarriage and from normal fertile women.

The basal production of LIF by human endometrial epithelial cells after 5 days in culture prepared from endometrial tissue obtained throughout the menstrual cycle from 21 different fertile individuals is shown in Figure 3. In these experiments the day of the cycle was calculated from the date of the last menstrual period. The results show that there is a variation in the amounts of LIF produced by the epithelial cells obtained at different times in the cycle. Significantly more LIF was produced by epithelial cells prepared from endometrium obtained in the late proliferative and early secretory phases of the cycle than by cells from the early proliferative ($P < 0.001$) and late secretory phases ($P < 0.01$) of the cycle. LIF was not detectable in the culture supernatants from stromal cells prepared from endometrial tissue obtained at any time in the cycle. Uptake of [3 H]thymidine into the cells showed that there was no difference in cell growth in cultures prepared from endometrium obtained at different times in the cycle.

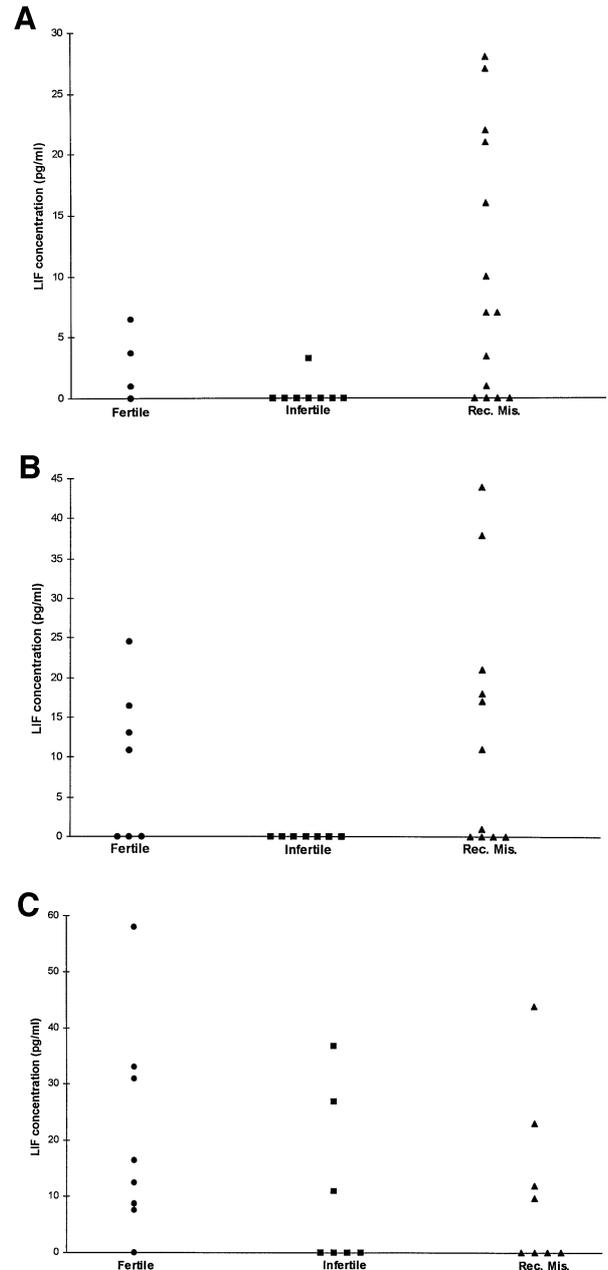


Figure 2. The concentrations of leukaemia inhibitory factor (LIF) in uterine flushings obtained on days LH+7 (A), LH+10 (B) and LH+12 (C) of the menstrual cycle from normal fertile women, women with unexplained infertility and women who suffer recurrent miscarriage. Concentrations of LIF in the flushings from women with unexplained infertility were significantly lower than in those obtained from normal fertile women on day LH+10 ($P < 0.05$); mean \pm SEM values (pg/ml) were: fertile 8.1 ± 3.3 , infertile 0.0 ± 0.0 . The detection limit for LIF was 2 pg/ml.

Figure 4 shows the effect of high doses of progesterone and oestradiol on the production of LIF by human endometrial epithelial cells in culture. Because of the variation in basal LIF production by cells obtained from different women at different times in the cycle, the data are shown as a percentage of the control values. Data from cells obtained from eight fertile women were normalized in this way. The secretion of LIF from these cells was decreased in the presence of oestradiol and progesterone; the effect of progesterone and oestradiol

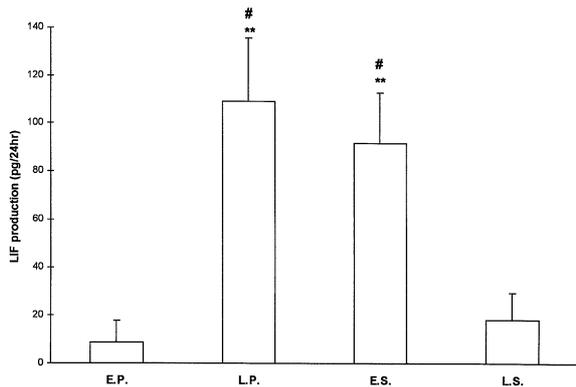


Figure 3. Basal leukaemia inhibitory factor (LIF) production by cultured human endometrial epithelial cells after 5 days in culture. The day of the menstrual cycle was dated from the start of the last menstrual period; $n = 3$ for early proliferative (EP), $n = 6$ for late proliferative (LP), $n = 6$ for early secretory (ES) and $n = 6$ for late secretory (LS). **Significantly different from EP (days 3–7) at $P < 0.001$; #significantly different from LS (days 15–22) at $P < 0.01$. Individual data points used to calculate the mean \pm SEM were obtained by averaging the amounts of LIF produced in the 15 control wells from each 96-well plate prepared from each biopsy sample (n).

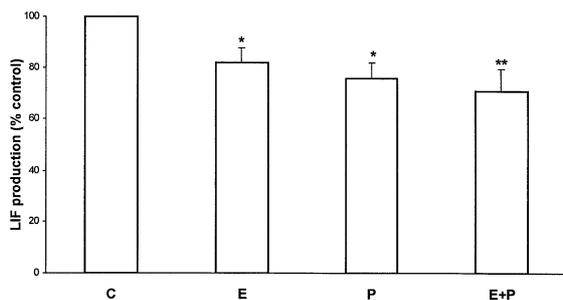


Figure 4. The effect of steroids on leukaemia inhibitory factor (LIF) production by cultured human endometrial epithelial cells. Values in the presence of steroids are expressed as a percentage of the control (C) values, mean \pm SEM for endometrial tissue obtained from eight individuals. E = oestradiol (10^{-6} M), P = progesterone (10^{-6} M), E+P = oestradiol plus progesterone (each at 10^{-6} M). **Significantly different from controls at $P < 0.05$ and $P < 0.01$ respectively.

together ($P < 0.01$) was greater than the effect of either steroid alone ($P < 0.05$). No difference in the effect of steroids on the response of cells prepared from proliferative and secretory endometrium was seen (data not shown).

Discussion

This study documents the first evidence for the production of LIF protein by human uterus *in vivo*. The finding of LIF in the flushings is strong evidence for its production by the endometrium *in vivo*. The flushings were obtained by washing out the endometrial cavity with saline, and factors within the cavity are most probably derived from the endometrium. This is supported by the cell culture experiments which show the capability of endometrial cells to produce and secrete LIF. The decrease in endometrial LIF production at the end of the cycle contrasts with that found for two other endometrial proteins, placental protein 14 and CA 125, whose concentrations con-

tinue to increase to the end of the cycle (Li *et al.*, 1993b; Dalton *et al.*, 1995). As with these previous studies, the amounts of LIF are expressed as an absolute concentration in the flushing and not per unit of protein. This reflects the differences in absolute amounts of LIF in the endometrial cavity at different times in the cycle. It is this amount of LIF that is relevant with respect to its biological action, regardless of the concentration of other proteins. The human embryo is thought to commence implantation on day LH+7. Thus the appearance of LIF in the uterine flushings coincides with the time of implantation and is similar to the time-course of expression of LIF in the mouse uterus (Bhatt *et al.*, 1991).

Although the population sampled was small, and some variation was seen in the concentrations of LIF found, the fact that LIF concentrations in flushings from women with unexplained infertility were significantly lower than those in flushings from normal fertile women is further evidence of a role for LIF in human embryo implantation. Our *in-vivo* data agree with the *in-vitro* studies of Delage *et al.* (1995), who showed that endometrial explant tissue prepared from women with unexplained infertility produced less LIF than tissue obtained from normal fertile women. For the group of infertile women included in this study it is unknown whether their endometrial histology is compatible with the timing of the LH surge. However, previous work has shown that unexplained infertility may be associated with an abnormal endometrial development, and this could be the reason for the decreased amounts of LIF in the flushings of these women. There was no difference in the amounts of LIF in the flushings of recurrent miscarriage and normal fertile women. The initial stages of embryo implantation in women who suffer recurrent miscarriage are thought to proceed normally, with miscarriage occurring later in the pregnancy. Taken together, these data support the view that LIF is an important mediator of early human embryo implantation.

The factors that control the production of LIF in human endometrium are unknown, but will be important in the understanding of the role of LIF in implantation. The control of LIF production by human endometrium is difficult or impossible to study *in vivo*, but can be studied using *in-vitro* cell culture of endometrial cells. In this study we used an established endometrial cell culture method to investigate LIF production *in vitro*. This culture system has been shown to be free of leukocytes which could contribute to LIF production (Laird *et al.*, 1993, 1994). The results of this study confirm previous reports showing that endometrial epithelial cells produce more LIF than stromal cells (Chen *et al.*, 1995). They also suggest that epithelial cells from late proliferative and early secretory phase endometrium produce more LIF than cells from early proliferative and late secretory phase endometrium. The uptake of [3 H]thymidine into the cells showed that these differences were not a result of differences in cell growth. Similar differences in LIF production by cultured epithelial cells obtained at different times in the cycle have been reported (Chen *et al.*, 1995). Menstrual dating for the endometrium used in the cell culture studies was taken from the time of the last menstrual period, which is less accurate than measurement of the LH surge. For samples obtained from hysterectomy

patients, the dating agreed with that obtained by histological observation of the endometrial tissue. For the normally cycling fertile women undergoing sterilization, their LH surge would be expected to fall on day 14 of their cycle. Even allowing for some variation in the dating of the endometrium used for the cell culture studies, the results suggest that the endometrial epithelial cells have the capacity to produce LIF prior to its appearance in the uterine flushings. This is not unexpected because it is likely that LIF is present in the flushings at an earlier time in the cycle, but is present below the level of detection of the assay. It is also likely that there will be a lag between the ability of the cell to produce LIF and its appearance in the endometrial cavity.

The effect of high doses of progesterone and oestradiol on LIF secretion by epithelial cells in culture was investigated. High doses of steroid were used in this study to overcome the binding of steroid to binding proteins present in the fetal calf serum, which sequester the steroid and make it unavailable to the cell. The inhibitory effect of steroids on LIF production seen in this study is difficult to relate to the increasing amount of LIF seen in the flushings in the secretory phase of the cycle. However, it is possible that LIF production *in vivo* is not under the direct control of steroid hormones. In-vivo LIF production may be controlled by the effect of steroids on factors produced from a different compartment of endometrial cells (such as stroma or leukocytes) which is not present in the cell culture system. Alternatively, the cells in culture may have lost their steroid receptors and are therefore responding to steroids in a non-physiological manner. There have been several reports published showing that human endometrial cells in culture do express steroid receptors (Iwai *et al.*, 1995; Zhang *et al.*, 1995). In addition, our previous work has shown that these endometrial cells in culture respond to progesterone and oestradiol in a physiological manner with respect to placental protein 14 production (Laird *et al.*, 1993). Therefore the loss of steroid receptor is an unlikely explanation for the lack of stimulatory response to steroid.

The mechanism of action of LIF in implantation is unknown. It is possible that LIF could exert its effect on the embryo and/or the endometrium. Both components of the LIF receptor have recently been shown to be expressed by human oocytes and human preimplantation embryos (Eijk *et al.*, 1996). In addition, LIF regulates the in-vitro production of urokinase plasminogen activator (uPA) and gelatinase B by peri-implantation embryos from mice (Harvey *et al.*, 1995). This suggests that LIF participates in the control of trophoblast invasion because uPA and gelatinase have been implicated in tissue invasion processes (Werb, 1989; Strickland and Richards, 1992). Little is known about the effects of LIF on endometrial function, although it has been reported that it has no effect on prostaglandin production by endometrial epithelial cells in culture (Chen *et al.*, 1995).

In summary, this study has shown for the first time the capability of human endometrium to produce LIF *in vivo*. The fact that maximum concentrations of LIF are seen at the time of implantation and that these are decreased in women with unexplained infertility suggests that LIF may be an important uterine factor in supporting implantation, as has been found

in the mouse. We have also shown that LIF production by human endometrial epithelial cells in culture varies depending on the time in the cycle that the tissue was taken. This variation *in vitro* is consistent with differences in LIF concentrations found in uterine flushings. However, the secretion of LIF by these cells was not stimulated by progesterone and oestradiol; instead these steroids caused a slight decrease in LIF production.

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