Leukaemia inhibitory factor mRNA concentration peaks in human endometrium at the time of implantation and the blastocyst contains mRNA for the receptor at this time

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Maternal endometrial leukaemia inhibitory factor (LIF) is required for successful implantation in mice. Mice with homozygous deletions in this gene fail to support implantation. The localization of immunoreactive LIF and the concentration of the mRNA encoding human LIF in normal endometrium during the menstrual cycle were investigated. The amount of RNA was low or undetectable in the proliferative phase but increased by approximately six times in the mid- to late secretory phase. The protein can only be detected by immunocytochemistry in glandular epithelium in the mid- or late secretory phase. To investigate the possible target for the endometrial LIF, we undertook reverse transcription—PCR analysis of early human embryos to determine whether they contain the mRNA encoding the LIF receptor. This study indicated that at the time of implantation in humans, the maternal endometrium produces LIF and that the blastocyst expresses LIF receptor mRNA and therefore may be capable of responding to this signal.

Introduction

Biologically active substances are named in a manner that relates to the bio-assay that was first used to isolate them. This was the case with leukaemia inhibitory factor (LIF). This secreted glycoprotein was first identified by its ability to induce differentiation and thereby inhibit the proliferation of the murine myeloid leukaemia cell line M1 (Hilton et al., 1988a). Since then, this factor has been re-isolated on the basis of a number of different bio-assays and consequently is known by a plethora of synonyms including: hepatocyte stimulating factor II and III (its action results in increased acute phase protein synthesis); cholinergic neuronal differentiation factor (induction of cholinergic phenotype); melanocyte-derived lipoprotein lipase inhibitor (inhibition of lipoprotein lipase) and differentiation—inhibitory activity (inhibition of embryonic stem cell differentiation). These data are reviewed by Hilton and Gough (1991). LIF can act on a wide variety of cells with apparently opposite effects in different cell types, in particular the ability to either promote or inhibit differentiation. Although the inhibition of leukaemic cell proliferation was the first action to be noted, it is the effect of LIF on embryonic stem cells that has recently come to the fore. This factor is required for the culture of embryonic stem cells and therefore plays an important role in the generation of so-called ‘knockout’ animals (Capecchi, 1989).

Despite the wide range of actions of this factor in vitro, its sites of production in vivo are only now being described (for example embryonic and adult brain, neonatal and adult heart; Patterson and Fann, 1992). In the adult mouse, the glandular epithelium of the uterine endometrium on day 4 of pregnancy (or pseudopregnancy) contains large amounts of the mRNA encoding LIF (Bhatt et al., 1991). Its expression is tightly regulated and is linked directly to the implantation of the mouse embryo. When a block causing natural or artificial delayed implantation is removed (thereby allowing implantation to occur), the content of endometrial LIF mRNA rises markedly suggesting that maternal LIF is required for implantation. This hypothesis is supported by the studies of Stewart et al. (1992), who showed, using targeted gene deletion, that maternal LIF is obligatory for the process of implantation in mice. We investigated whether the mRNA encoding LIF is expressed in human endometrium during the menstrual cycle and in particular whether the amount of this mRNA rises at the time of implantation.

The biological action of LIF is mediated by both high- and low-affinity receptors (Kd value 10–200 pmol l⁻¹; Yamamoto-Yamaguchi et al., 1986; Hilton et al., 1988b; Kd value 1–3 nmol l⁻¹; Hilton et al., 1991). Solubilization of membranes containing the high-affinity class results in their conversion to the low-affinity type. This finding suggests that there are two or more subunits in the high-affinity class, one of which upon disaggregation retains low-affinity binding (Nicola and Metcalf, 1991). Two such subunits have been cloned and transfection of these cDNAs at least mimics what is observed in normal cells. The low-affinity subunit is converted to the high-affinity type by dimerization with the gp130 signal transducing unit also found in the IL-6 receptor. Thus it seems that both LIF and IL-6 share sequence similarity and their mechanisms of receptor action are also similar (Gearing et al., 1991, 1992).
Here we report on the temporal regulation of the mRNA encoding LIF during the menstrual cycle and the localization of LIF immunoreactivity in the human endometrium. We also demonstrate the presence of mRNA encoding the LIF receptor on preimplantation human blastocysts. These data suggest that LIF is produced by the human glandular endometrium in the mid-secretory phase when implantation occurs, and that the embryo can respond to this maternal signal.

Materials and Methods

Tissue collection and RNA extraction

Endometrial tissue was removed at dilatation and curettage or hysterectomy, performed for benign gynaecological conditions. The majority of patients were undergoing dilatation and curettage for the investigation of subjective menorrhagia. The tissue obtained at this time was shown to be histologically normal. It has further been shown that at least 50% of women complaining of menorrhagia in fact have blood loss values within the normal range (Cameron et al., 1990). Tissue obtained from most of these women would therefore have been normal. Informed consent was obtained from the patients and the study passed by the ethical committee of the Cambridge District Health Authority.

Human preimplantation embryos (blastocyst stage) produced by in vitro fertilization and which had been donated for research were obtained from the Bourn Hall Clinic. All procedures involving human embryos were carried out in accordance with the regulations of the Human Embryology and Fertilization Authority (HEFA).

Preparation of total RNA

Immediately on collection, tissue samples were flash-frozen in liquid nitrogen. Tissue was stored at −70°C until used. Total RNA was isolated by the method of Chomczynski and Sacchi (1987) in which frozen tissue is homogenized in 5 ml buffer containing 4 mol guanidinium thiocyanate 1⁻¹ (Gibco BRL Livingston, Irvine), 25 mmol sodium citrate 1⁻¹ pH 7.0, 0.5% sarcosyl and 0.1 mol 2-mercaptoethanol 1⁻¹. The lysate was acidified by the addition of 0.5 ml 2 mol sodium acetate 1⁻¹ pH 4 and phenol—chloroform extracted using 5 ml of buffer-saturated phenol and 1 ml chloroform—isoamyl alcohol (49:1 v:v). The suspension was placed on ice for 15 min and centrifuged at 10 000 g for 20 min at 4°C. The aqueous phase containing RNA was precipitated by addition of isopropanol and incubation at −70°C. RNA was recovered by further centrifugation, desiccated and redissolved in homogenization buffer. Finally, RNA was precipitated, washed twice in 70% ethanol, dried and resuspended in TE (10 mmol Tris—HCl 1⁻¹ pH 7.4 and 1 mmol EDTA 1⁻¹). The concentration of RNA was determined by spectrophotometry at 260 nm.

RNA was prepared from single human embryos using a scaled down protocol based on the above procedure. A single embryo was flash-frozen in an Eppendorf tube (and thus lysed by ice crystals). Precipitation of the RNA was assisted by adding 100 µg of carrier yeast tRNA (Gibco BRL) at the homogenization step. The remaining details are as described above except that all the volumes were 50 times less and the whole procedure was carried out in 400 µl Eppendorf tubes.

RNAse protection assay

The RNAse protection assay was based on solution hybridization between the mRNA of interest and a labelled antisense RNA probe. The specific hybrid was detected in a quantitative manner after the digestion of all the remaining single-stranded RNA (both the excess probe and cellular RNA) by a single-strand specific RNAse. The probe used in this study was a genomic fragment obtained by PCR which was cloned in the plasmid vector pBluescript (Stratagene, La Jolla, CA); this was digested within the third exon with Sty I and the antisense RNA transcribed in vitro from the T7 promoter. This resulted in a probe of 399 bases which contained bases 378—703 (based on the numbering of the cDNA sequence) and 74 bases transcribed from the vector. The transcription reaction (20 µl), contained 1 µg linearized DNA template, 1 mmol 1⁻¹ ATP, GTP and CTP 2.5 µmol 1⁻¹ unlabelled UTP, 60 µCi [α³²P]UTP (800 Ci mmol⁻¹, Amersham, Bucks), 20 U human placental ribonuclease inhibitor (HT Biotechnology, Cambridge, UK), 20 U T7 RNA polymerase (Ambion, Dallas, TX). After incubation for 1 h at room temperature 2 U RNAse-free DNase was added and the reaction incubated at 37°C for 15 min to digest the DNA template. This resulted in synthesis of a probe with a specific activity of approximately 8 x 10⁸ d.p.m. µg⁻¹. The probe was then purified by preparative PAGE. The full length probe was eluted from the acrylamide and its concentration determined by liquid scintillation counting of a small portion. Excess probe (2—4 x 10⁶ c.p.m.) was then coprecipitated with each sample of endometrial RNA (30 µg) and resuspended in hybridization buffer (RP11 kit Ambion). After heat denaturation (95°C for 3 min), hybridization was allowed to occur by incubation for 16—20 h at 42°C. The excess probe was digested by RNAse A and the protected hybrid was recovered by ethanol precipitation. The entire sample was then loaded on a denaturing 6% polyacrylamide sequencing gel. Detection of a protected fragment of 325 nucleotides indicated the presence of the mRNA encoding LIF in the cellular RNA sample.

Immunocytochemistry

Tissue for immunocytochemistry was fixed in periodate—lysine—paraformaldehyde (PLP) fixative for 8 h at room temperature, followed by routine wax processing (without formalin). Serial sections (10 µm) for immunocytochemistry were dewaxed in xylene, and rehydrated through an alcohol series. Endogenous peroxidase activity was destroyed by incubation in PBS with 3% hydrogen peroxide. Blocking was carried out with 10% horse serum in PBS followed by incubation with goat anti-hLIF antibody (British Biotechnol¬ogy, Abingdon) for 1 h at room temperature. This was detected with biotinylated horse anti-goat antibody, followed by ABC peroxidase (Vectastain, Peterborough). Irrelevant isotype-matched primary antibodies, and normal goat serum were used as negative controls. Sections were counterstained with haemalum and mounted in Depex (BDH, Poole).
LIF expression in endometrium

Polymerase chain reaction

Human embryos of normal appearance under the microscope and which had been fertilized as part of an IVF programme were obtained from Bourn Hall. These embryos had been donated for research purposes by the parents and this study complied with the requirements of the Human Embryology and Fertilization Bill.

Total RNA was prepared from frozen human embryos by the scaled down version of the method described above. cDNA was synthesized using AMV reverse transcriptase (Super RT, HT Biotechnology, Cambridge). Total RNA extracted from one embryo in 5 µl TE was primed with oligo dT (Pharmacia, Milton Keynes), according to the manufacturers' instructions for 60 min at 42°C. For initial amplification by PCR, 10% of the reverse transcriptase products were amplified using primers 1 and 2 with Amplitaq (Cetus, Emeryville), in the manufacturers' recommended buffer. The primers were designed to amplify a 458 base pair (bp) fragment of the cDNA for the LIF receptor. After 30 cycles of PCR using primers 1 and 2, 2% of the first round reaction was transferred to a fresh tube containing the inner primers 3 and 4, and subjected to a further 30 rounds of amplification. As negative control, an equal volume of the culture fluid in which the embryo was grown was extracted in the same way. In addition, 200 cells of the BeWo cell line were extracted as a positive control. Contamination of the RNA by genomic DNA was identified by subjecting 1 µg of genomic DNA to PCR at the same time as the cDNA. The primers used in this study are specific to the LIF binding subunit of the LIF receptor (i.e. not the gp130 affinity converter described by Gearing et al., 1992) and their sequences are shown below. Numbering of bases is according to Gearing et al., 1991.

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\begin{align*}
\text{Bases} & & \text{5'} & & \text{3'} \\
1 & (2807 \text{ to } 2826) & \text{GAAAACTGTAAAGCATTACA} \\
2 & (-3310 \text{ to } -3292) & \text{AGAGTTGGAGAAGTACTAA} \\
3 & (2831 \text{ to } 2848) & \text{CAAAGATGCGTCTGAG} \\
4 & (-3289 \text{ to } -3272) & \text{CCATGTATTTACATTGGC}
\end{align*}
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Results

LIF mRNA concentration

The endometria were dated using the criteria of Noyes et al. (1950). Equal amounts (30 µg) of each RNA was analysed using the RNAse protection assay to detect and quantitate mRNA encoding LIF. This assay allows quantitative comparisons to be made between samples analysed in parallel and run on the same gel (Fig. 1a). The autoradiographic signal arising from the protected fragment was analysed by densitometry. If the data are plotted with respect to the stage of the menstrual cycle (Fig. 1b), there is a peak in the amount of LIF mRNA in the late secretory phase of the cycle. This is at least six times higher than that detected in the proliferative phase when the amount of LIF is low or undetectable.

Immunocytochemistry

Fixed sections of endometrial tissue were analysed using a polyclonal antiserum raised against recombinant human LIF. There was no staining of either the glandular epithelium or the stromal tissue in the proliferative phase of the menstrual cycle (Fig. 2c, d). Samples from six different patients were analysed for each phase of the cycle. Tissue obtained in the mid- and late luteal phase shows clear and reproducible staining in the glandular epithelium (Fig. 2e, f). There was also faint staining in the stroma in the luteal phase; however, this was weak and appeared to be primarily associated with the extracellular matrix. Blood vessels in the endometrium occasionally showed weak LIF immunoreactivity at all stages of the cycle. However, in the myometrium there was strong staining of both the media of the larger blood vessels and the majority of the smooth muscle cells (Fig. 2a, b). The RNA encoding LIF has also been detected in myometrium using RT–PCR as well as the RNAse protection assay (data not shown).

Embryo LIF receptor PCR

Total RNA was prepared from three blastocysts pooled together and an equal volume of embryo culture fluid in parallel (the latter serving as a negative control). Half of the RNA was

Fig. 1. (a) Autoradiograph, and (b) plot of the relative signal intensity, from the RNAse protection assay that specifically detects the mRNA encoding human leukaemia inhibitory factor (LIF). The samples have been arranged according to histological dating of the endometrium. Densitometric analysis was carried out using a Molecular Dynamics (Sunnyvale, CA) laser densitometer.
used as the template for cDNA synthesis as described by Haining et al. (1991). This was then amplified using the nested PCR primers described above which are specific for the mRNA encoding the high-affinity portion of the LIF receptor. A single band of the predicted size was observed following agarose gel electrophoresis (Fig. 3, lane e). The identity of this band was confirmed by cloning and sequencing and it was identical to the published sequence of the LIF receptor. The negative controls used were amplification of ‘extracted’ embryo culture fluid or amplification without added template. No DNA product was observed in these lanes indicating that there was no contamination with extraneous cDNA or RNA. The amplification of the cDNA obtained from 200 BeWo cells showed specific bands corresponding to both genomic DNA and the cDNA product. PCR with the LIF-specific primers using genomic DNA as the template resulted in an amplified product of 1.2 kb as opposed to the 458 bp product from the cDNA. This difference is attributable to the presence of an intron between bases 246 and 247 in the cDNA. Thus the presence of spliced RNA can be unambiguously detected.

Fig. 2. Immunocytochemical localization of leukaemia inhibitory factor (LIF) immunoreactivity in human myometrium (a and b), proliferative endometrium (c and d) and secretory endometrium (e and f). (a), (c) and (e) show staining with normal goat serum and do not show any specific staining. Panels b and f show staining (brown) with the anti-LIF antisera in myometrium and secretory phase endometrium, respectively. (d) shows that LIF immunoreactivity is absent from proliferative phase endometrium. LIF immunoreactive cells can be seen in the smooth muscle cells and in the media around blood vessels (b) and glandular epithelium of the secretory phase endometrium (f). Sections were counterstained with haemalum. The scale bar represents 100 µm in (a) and (b) and 50 µm in (c), (d), (e) and (f).
Fig. 3. Agarose gel showing the reverse transcription (RT)–PCR amplified products from RNA derived from preimplantation human embryos using leukaemia inhibitory factor (LIF) receptor specific primers. Lane e: RNA from three blastocyst stage embryos; lane s: embryo culture medium supernatant control; lane c: control (no RNA); lanes b: RNA from 200 BeWo choriocarcinoma cells; lane m: molecular weight markers (123 bp ladder, Gibco BRL, in which each band is 123 bp longer than the band below it).

**Discussion**

Maternal production of LIF is an essential requirement for implantation in mice. Female mice with homozygous deletions in the LIF gene are infertile because embryos (normal or mutant) cannot implant. Embryos that carry this deletion can implant when transferred to normal dams (Stewart et al., 1992). Although these data clearly show the requirement for maternal LIF, it is not clear whether LIF acts directly on the embryo or serves to effect a maternal response.

Implantation in the mouse occurs at 4.5 days postcoitum and expression of LIF mRNA is highest on day 4 (day 1: the day of the vaginal plug). When delayed implantation occurs, the burst of LIF expression is also delayed and remains closely linked to the time of implantation (Bhatt et al., 1991). We investigated the temporal regulation of human endometrial LIF expression. Clearly the human reproductive cycle is different from that observed in mice. However, the endometrium in the mid- to late secretory phase of each cycle is prepared for implantation, which would be expected to occur at this time in a conception cycle. Thus, if the action of LIF is similar in mice and humans, LIF production would be expected to be maximal at this time. Using a specific RNAse protection assay, we showed that the amount of mRNA encoding LIF is highest at this time. The tissue samples used in this study were dated by histology and not by reference to the LH surge. This may account for the slight variations observed in LIF mRNA content between samples at apparently the same stage of the cycle. Equally the variation may reflect differences present in a natural population.

LIF immunoreactivity was localized in the glandular epithelium of the mid- and late secretory phase of the menstrual cycle, but was undetectable in proliferative endometrium, suggesting that the protein is produced only at the former time in these cells. Some LIF immunoreactivity was detected in the stroma, but this was weak and variable in comparison to that in the glands. LIF binds to the extracellular matrix (Rathjen et al., 1990) and we observed some weak matrix-associated staining. This is the first localization of LIF immunoreactivity in normal human tissue. It is in concordance with the assessment of the steady state LIF mRNA content in endometrium (determined by the RNAse protection assay) and therefore confirms the local production of LIF by human endometrium in the secretory phase. The observed increase in LIF mRNA concentration in the luteal phase of the cycle may be attributable to increased transcription in the glands or may simply result from the increase in the proportion of glandular epithelium in the endometrium at this time. Either mechanism would be expected to result in increased amounts of LIF in the uterine lumen. Since LIF immunoreactivity appears as its mRNA becomes detectable, it is probable that LIF production is transcriptionally regulated in the epithelial cells. The finding that LIF immunoreactivity and mRNA are both present in human myometrium is also novel and may indicate that LIF has a role in this tissue. LIF has already been shown to promote myoblast proliferation *in vitro*, an effect that is enhanced by other cytokines (Austin et al., 1992).

Since LIF mRNA and protein content are highest at about the time of human implantation, it is probable that LIF has a similar role in humans to that observed in mice. However, what this role is remains to be resolved.

In light of the effect of LIF on embryonic stem cells, it is possible that *in vivo* LIF may act directly on the embryo. LIF has also been shown, using *in vitro* differentiating mouse embryonic stem cells (embryoid bodies), to permit primitive endoderm formation but to inhibit primitive ectoderm differentiation (Shen and Leder 1992). To investigate this further, we sought evidence that the human preimplantation embryo could respond to exogenous LIF. The RT–PCR of early embryo RNA clearly demonstrates that such embryos at least have the capacity to synthesize the LIF receptor and it is possible that maternal LIF is signalling the embryo in advance of or during implantation.

This is the first report of the presence of LIF and its receptor in human endometrium and embryo, respectively. Further work is needed to elucidate the factors that control LIF production and more importantly to characterize the target cells and their responses to LIF stimulation. As the experiments performed in mice by Stewart et al. (1992) have shown, this factor is of central importance in reproductive biology and therefore warrants further investigation in humans.

The authors would like to thank the Consultant and theatre staff of Addenbrookes’ Hospital, Cambridge for their help with this study and A. King for assistance with the endometrial dating. D. S. Charnock-Jones was supported by MRC project grant G892883/EB, A. M. Sharkey by MRC project grant 89099705/EB.
References


Hilton DJ, Nicola NA, Gough NM and Metcalf D (1988a) Resolution and purification of three distinct factors produced by Krebs ascites cells which have differentiation-inducing activity on murine leukemia cell lines. *Journal of Biological Chemistry* 263 9238–9443

Hilton DJ, Nicola NA and Metcalf D (1988b) Specific binding sites of murine leukemia inhibitory factor to normal and leukemic monocyte cells. *Proceedings National Academy Sciences USA* 85 5971–5975


Noyes RW, Hertig AT and Rock J (1950) Dating the endometrial biopsy. *Fertility and Sterility* 1 3–25

Patterson PH and Fann MJ (1992) Further studies of the distribution of CDF/LIF mRNA, in Polyfunctional cytokines IL-6 and LIF. (CIBA Foundation Symposium) 167 125–135


Shen MM and Leder P (1992) Leukemia inhibitory factor is expressed by the preimplantation uterus and selectively blocks primitive ectoderm formation in vitro. *Proceedings of the National Academy Sciences USA* 89 8240–8244
