Trophic effects of myeloid leukaemia inhibitory factor (LIF) on mouse embryos

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Myeloid leukaemia inhibitory factor (LIF) is expressed at highest concentrations in the maternal endometrial glands at about the stage of blastocyst implantation. LIF is also expressed by the extraembryonic membranes of the early mouse embryo. Embryos of different ages were cultured with, or without, LIF, and embryo growth *in vivo* and *in vitro* was examined to determine whether LIF is important for embryo development. Supplementing embryo culture media with 1000 U recombinant human LIF ml⁻¹ increased the number of eight-cell mouse embryos developing beyond the hatched blastocyst stage *in vitro* from 62.1% to 85.1% ($P < 0.05$). LIF significantly increased the number of embryos hatching (33.8% versus 7.65% for controls 96 h after hCG injection, $P < 0.001$), completely hatching (85.1% versus 62.1%, $P < 0.05$), and exhibiting trophoblast outgrowth (13.5% versus 0% 120 h after hCG treatment, 85.1% versus 47.0% 144 h after hCG treatment, $P < 0.001$) *in vitro*. LIF-treated embryos also displayed a significantly greater area of trophoblast outgrowth than did controls as early as day 5 in culture ($P < 0.005$). These data show that LIF enhances mouse eight-cell embryo development *in vitro*, as seen by the accelerated rate of embryo hatching and trophoblast outgrowth. In addition, enhanced embryo survival *in vivo* is shown, following the transfer of LIF-treated embryos into a pseudopregnant recipient female. Expression of mRNA encoding LIF was detected in endometrial cells cultured in monolayer from uteri of day 3 pregnant females, explaining the known embryotrophic effects of endometrial coculture. This expression was not enhanced significantly by treatment with oestradiol (3.7×10^{-5} mol l⁻¹) or progesterone (3.2×10^{-6} mol l⁻¹) or both hormones. These results indicate that LIF could have a dual action in early embryogenesis as an embryotrophin and as a factor required for embryo implantation. Multiple roles for LIF are consistent with the expression of this factor at embryonic, extraembryonic and maternal sites during early embryogenesis.

Introduction

The establishment of an appropriate uterine environment during mammalian embryogenesis depends upon complex interactions between embryo and uterus. An increasing body of evidence indicates that peptide-signalling molecules play an important role in this process. These include proteins originally identified as growth factors (see reviews by Hill *et al.*, 1987 and Brigstock *et al.*, 1989) and proteins such as cytokines that were identified in other cellular systems. These molecules are located in the uterus during pregnancy and have been shown to regulate the attachment, implantation, growth and development of embryo and fetus (Pollard *et al.*, 1987; Wegmann, 1988; Arceci *et al.*, 1989; Wegmann *et al.*, 1989; Pollard, 1990; Robertson *et al.*, 1990).

Myeloid leukaemia inhibitory factor (LIF) has pleiotropic activities in a number of mammalian cell systems (Hilton *et al.*,

1988; Moreau *et al.*, 1988; Smith *et al.*, 1988; Williams *et al.*, 1988; Gough *et al.*, 1989; Metcalf 1989; Yamamori *et al.*, 1989). Several lines of evidence indicate that this protein has important biological activities during early mammalian development (see review by Fry, 1992). LIF is expressed at a variety of sites in both the mother and the early conceptus (Robertson *et al.*, 1993), and has potential biological effects on several cell types in the early embryo. In particular LIF is expressed by the maternal oviduct and uterus, and at high concentrations in the endometrial glands about the stage of implantation (Bhatt *et al.*, 1991; Shen and Leder, 1992). Embryonic LIF expression has been observed in the extraembryonic membranes of the egg cylinder embryo and continues after gastrulation (Conquet and Brûlet, 1990; Rathjen *et al.*, 1990a). *In vitro*, LIF can prevent the differentiation of embryonic stem cells (Smith *et al.*, 1988; Williams *et al.*, 1988) which are derived from, and closely related to, cells of the embryonic inner cell mass. Receptors for LIF have also been identified on trophectoderm cells (Fry, 1992). Genetic experiments have indicated that overexpression of LIF in chimaeric embryos can inhibit gastrulation (Conquet

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et al. 1992), while mutation of the LIF gene in female mice prevents embryo implantation (Stewart *et al.*, 1992). Although the requirement for maternal LIF expression for embryo implantation is well defined, the reasons for the multiple sites of LIF expression during early embryogenesis and potential effects of the cytokine on the embryo at this stage are not clear.

The aim of this study was to explore the possible role of LIF in early embryonic development by analysis of the effects of LIF on embryo development, growth and implantation *in vitro*.

We have previously shown (Lavranos and Seamark, 1989) that mouse embryo development can also be enhanced by coculture with endometrial cells in the presence of oestradiol and progesterone. To test whether this effect was associated with steroid-induced production of LIF by endometrial cells, these cells were cultured in the presence or absence of steroids and examined for the expression of mRNA encoding LIF by ribonuclease protection assays.

Materials and Methods

Animals

Female mice 3–4 weeks-old (F_1 : BALB/c $\times$ C57Blk strain or CBA $\times$ C57 strain as indicated) were given an i.p. injection of 7.5 iu pregnant mares' serum gonadotrophin (Folligon; Intervet Pty Ltd, Sydney) and 7.5 iu hCG (Chorulon; Intervet Pty Ltd) 48 h later to achieve superovulation. After hCG treatment, each female was placed with a fertile male mouse (CBA $\times$ C57) and left overnight to mate. Each morning after males and females had been caged together, each female mouse was examined for evidence of mating; those with a vaginal plug were considered to be at day 1 of pregnancy, or day 1 of pseudopregnancy. Pseudopregnant female mice, to be used as recipients in embryo transplantation (embryo transfer) experiments, were prepared by mating with vasectomized males (CBA $\times$ C57).

Collection and culture of embryos

Pregnant females were killed by cervical dislocation either 20 h after the hCG injection for the collection of one-cell embryos or 71–73 h after the hCG injection for the collection of eight-cell embryos. The whole reproductive tract was dissected out and placed in equilibrated (with 5% CO₂ in air) Earle's balanced salt solution without calcium or magnesium (EBSS) at 37°C.

One-cell embryos were recovered from the ampulla of day 1 pregnant CBA $\times$ C57 females (4–6 females per replicate), washed twice in culture medium and randomly assigned to either a control or experimental group within 20 min of collection. All one-cell embryos were cultured in human tubal fluid medium (HTF) which was prepared according to Quinn *et al.* (1985). Each group consisted of approximately 10–15 embryos placed in a 20 μ l drop of either (i) HTF-unsupplemented medium (control group) or (ii) LIF-supplemented HTF medium, the concentration of LIF varying from 62.5 U ml⁻¹ to 2000 U ml⁻¹. Both control and experimental groups were repeated six times. Embryos were cultured for approximately 5 days in a drop of medium under paraffin

oil in an incubator at 37°C, in a humidified atmosphere of 5% CO₂ : 5% O₂ : 90% N₂. Development of the embryos was assessed as described below.

Eight-cell embryos were teased or flushed out of the oviduct–uterus junction of day 3 pregnant BALB/c $\times$ C57Blk females (4–6 females per replicate), washed twice in culture medium and randomly allocated to either a control group (unsupplemented culture medium) or experimental group (LIF-supplemented culture medium, 1000 U ml⁻¹) within 20 min of collection. The culture medium was prepared as described by Lavranos and Seamark (1989). Briefly, the culture medium was filter-sterilized minimal essential medium (MEM; Eagle, with Earle's salts, with L-glutamine, without sodium bicarbonate; Flow Laboratories, Herts) supplemented with 5.6 mmol glucose l⁻¹, 25 mmol sodium bicarbonate l⁻¹, 10% (v/v) heat-inactivated fetal calf serum, penicillin/streptomycin/fungizone (10 000 units, 10 000 μ g and 25 μ g, respectively, per 100 ml of medium) (CSL, Parkville, Victoria) and the pH and osmolality of the medium adjusted to 7.40 and 280 mosmol, respectively. Embryos (10–20) were cultured in 1 ml medium per well (four-well multidish; Nunc, Roskilde), 1–2 wells per replicate, 4–8 replicates per group (according to the experiment). All eight-cell embryos were cultured for 5–7 days in an incubator at 37°C, in a humidified atmosphere of 5% CO₂ in air, and growth and development were observed, and assessed as described by Lavranos and Seamark (1989). Briefly, the numbers of embryos achieving morula, blastocyst or hatching blastocyst stage as described by Hsu (1979) were recorded daily using an inverted microscope.

Embryo transfers

The procedure followed for embryo transfers was a modification of the protocol described by Hogan *et al.* (1986). Once collected, eight-cell embryos were cultured in either unsupplemented medium (controls) or in LIF-supplemented medium (1000 U ml⁻¹) until they reached either the hatching-blastocyst stage or the hatched-blastocyst stage. At each of these stages, four embryos from the control group were transferred to one uterine horn of a day 2.5 pseudopregnant recipient female, and four embryos from the LIF-treated groups were transferred to the contralateral uterine horn of the same animal; 13 females were used for hatching-blastocyst stage embryos and eight females were used for hatched-blastocyst stage embryos. Pseudopregnant females were anaesthetized for the surgical transfer of embryos by i.p. injection of Avertin (0.14 ml/10 g; Winthrop Laboratories, Ermington, NSW). Embryos to be transferred were loaded into a pulled sterile glass pipette and placed to one side until the time of transfer. The pseudopregnant recipient mouse was then placed under a dissecting microscope dorsal surface uppermost and either side of the lower back was plucked free of fur and swabbed with alcohol. A small (1.5 cm) mid-dorsal incision was made, the ovary located through the body wall and another small incision made avoiding any blood vessels. Part of the reproductive tract was pulled to the outside; the oviduct–uterine junction was located; the pipette containing the embryos was carefully inserted into the uterine lumen and the embryos were expelled. This was carried out for both sides of the reproductive tract.

Once both sides of the mouse had been used for embryo transfer, the incisions were sutured and the mouse left to recover. The survival rate on the recipient animals following this procedure was 100% (23 of 23), with a pregnancy success rate of approximately 90% (21 of 23). The recipient mice were allowed to reach day 15 of pregnancy at which time they were killed by cervical dislocation. Uterine horns from each female were assessed for the number of embryos either reabsorbed or the number of live fetuses.

Morphological assessment of embryo development

All embryos were observed daily, using an inverted microscope, and details of their development recorded during culture for 5–7 days. The numbers of embryos reaching various stages from one-cell for one group of experiments, or eight-cell through to hatching-blastocyst stage (Hsu, 1979) and their rates of development following hCG treatment, were recorded. Further development of these embryos related to implantation was also monitored (Sherman, 1978). On the basis of assessment of morphological development of mouse embryos after the blastocyst stage, as described by Lavranos and Seamark (1989), post-hatching embryos with proliferating trophoblast cells were recorded as attaining stage 1, and those with trophoblast cells attached to the bottom of the dish classed as stage 2. Embryos with trophoblast cells spread out on the bottom of the dish were classed as stage 3. The survival rate was calculated as the percentage of embryos that attained stage 3; the morphological equivalent *in vitro* of implantation. Quantitative measurements of stage 3 embryos were made using the Bio-Quant System image analyser and a Leitz microscope.

Expression of LIF in cultures of mixed uterine endometrial cells in the presence or absence of steroids

The procedure used for the preparation of mixed monolayers from whole uteri previously described by Salomon and Sherman (1975) and following the modifications of Lavranos and Seamark (1989) was as follows: after collection of the embryos from females at day 3 of pregnancy, uteri were dissected free of remaining oviductal and fatty tissue and washed twice in EBSS at 37°C under sterile conditions. They were then cut into smaller pieces (approximately 3 mm long) and placed in a Petri dish containing 0.5% (v/v) trypsin and 0.02% (v/v) EDTA in EBSS, for 15 min at 37°C. The enzyme solution was then replaced with fresh enzyme solution, the uterine tissue dissociated by mincing with sterile scissors for about 1 min and the mixture incubated at 37°C for 30 min. After the second enzyme treatment, heat-inactivated fetal calf serum (5%) was added and the cell suspension centrifuged at 500 g for 20 min, the supernatant discarded and the pellet resuspended in culture medium supplemented with or without steroid hormone where indicated. Cells were plated out into four-well multidishes (Nunc) at 1.3×10^6 cells per well and the dishes placed in a 37°C incubator in a humidified atmosphere of 5% CO₂ in air. Cells were cultured for 48 h in the presence, or absence, of steroid hormones; oestradiol (3.7×10^{-5} mol l⁻¹), progesterone (3.2×10^{-6} mol l⁻¹) or both steroid hormones

together (Lavranos and Seamark, 1989). The cells were then collected by treatment with 0.05% (v/v) trypsin and 0.02% (v/v) EDTA in EBSS, centrifuged at 500 g for 10 min; the supernatant was aspirated and discarded; and the tubes containing cells stored at -80°C for subsequent extraction of RNA. This experiment was repeated twice. Total RNA from each group of cells was assayed for LIF expression by ribonuclease protection assays.

RNA analyses

Total RNA was isolated as described by Edwards *et al.* (1985). Ribonuclease protection assays were carried out essentially as described by Rathjen *et al.* (1990a). Total RNA (5 µg) was hybridized with probe in the absence of added tRNA. Hybridizations were carried out at 45°C for 16–20 h. RNA hybrids were digested with 40 µg RNAaseA ml⁻¹ (Boehringer Mannheim, Mannheim) and 2 µg RNAase T1 ml⁻¹ (Bethesda Research Laboratories, Bethesda, MD) at 4°C for 30 min. Protected fragments were analysed on a 5% polyacrylamide–8 mol urea l⁻¹ gel, which was dried before autoradiography. High specific activity riboprobes for LIF were synthesized by transcription of pDR2 *in vitro* (Rathjen *et al.*, 1990b) with T7 polymerase (Bresatec, Adelaide) after linearization of the template by digestion with *Hind*III (Rathjen *et al.*, 1990b). The reaction conditions were as described by Krieg and Melton (1987) with the exception that 250 µCi [α -³²P]UTP (3000 Ci mmol⁻¹, 10 mCi ml⁻¹; Bresatec) was used in a 15 µl reaction volume. When using LIF clone pDR2 (Rathjen *et al.*, 1990b) two RNAase protected fragments are produced. The larger fragment (368 nucleotides) corresponds to the diffusible form of LIF and the shorter fragment (345 nucleotides) to the matrix form of LIF, produced by the use of alternative exons at the 5' end (Rathjen *et al.*, 1990b).

The concentrations of mouse glyceraldehyde-3-phosphate dehydrogenase (mGAP) mRNA were also determined and used as a control to show that equal amounts of total RNA had been analysed from each sample. The GAP riboprobe was synthesized in a 15 µl reaction volume containing 5 µCi [α -³²P]UTP (3000 Ci mmol⁻¹, 10 mCi ml⁻¹, Bresatec) and 3 µl of 1 mmol UTP l⁻¹ to reduce overexposure of the GAP signal relative to the LIF signal. This yielded probes of 500-fold lower specific activity than the LIF probes. The GAP signal consists of a triplet of protected bands at approximately 250 bp. An error (orientation of insert) was made in the original description of the preparation of the GAP probe (Nomura *et al.*, 1989). Antisense RNA was generated using SP6 transcription of *Bam*HI-linearized plasmid, and the three protected bands in mouse RNA represent heterogeneity at the 5' end as reported previously (Rathjen *et al.*, 1990a).

Statistical analyses

Results of embryo development were generally analysed by chi-squared tests. The data on the area of embryo inner cell mass and trophoblast following culture *in vitro* with LIF were analysed using Student's *t* test.

Table 1. The effect of myeloid leukaemia inhibitory factor (LIF: 1000 U ml⁻¹) on the rate of hatching of mouse embryos *in vitro*

Treatment	n	Percentage hatching ^a			Percentage hatched
		96 h	100 h	120 h	
Control	66	7.6	68.9	70	62.1
LIF	74	33.8***	93.2***	98.6	85.1*

^aTime after hCG injection.

Asterisks indicate significant differences from respective control groups:

P* < 0.05; **P* < 0.001.

Results

Effects of LIF on the development of one-cell embryos

When the medium was supplemented with a range of concentrations of LIF (62.5 U ml⁻¹ to 2000 U ml⁻¹) the results obtained (data not shown) did not differ from those obtained for LIF at 1000 U ml⁻¹. The addition of LIF (1000 U ml⁻¹) did not significantly alter the proportion of embryos developing from the one-cell stage through to the blastocyst stage, compared with the unsupplemented group (84.1% or 58/69 versus 90.1% or 64/71, respectively). After daily observation and assessment of the rate of growth and development, no significant differences in either the rate of development or the total number of embryos reaching a particular stage of development were evident between the unsupplemented and LIF-supplemented groups throughout the culture period. Thus LIF appeared to have no effect on development of embryos from the one-cell stage.

Effects of LIF on the development of eight-cell embryos

Robertson *et al.* (1990) found that culture of eight-cell embryos in medium supplemented with LIF (1000 U ml⁻¹) for a period of 5 days significantly increased (*P* < 0.01) the total number of embryos exhibiting trophoblast outgrowth *in vitro* from 64% (223 of 349) to 80% (156 of 195) of eight-cell embryos cultured in unsupplemented medium. The experiments reported here, using a concentration of LIF of 1000 U ml⁻¹, found that significantly (*P* < 0.001) greater proportions of LIF-treated embryos were hatching 96 h after hCG treatment (33.8%) when compared with the unsupplemented group (7.6%), suggesting that LIF had accelerated their rate of development. Furthermore, the total number of LIF-treated embryos that completely hatched (85.1%) was significantly (*P* < 0.05) greater than the number observed in the unsupplemented group (62.1%) (Table 1). Continued observation and assessment of embryo growth and development revealed that a significant (*P* < 0.01) proportion (13.5%) of LIF-treated embryos had attained stage 2 (trophoblast cells attached to the bottom of the dish) 120 h after hCG treatment compared with none in the unsupplemented group (Table 2). By 144 h after hCG treatment, trophoblast outgrowth was complete (stage 3) in 85.1% of LIF-treated embryos compared with only

Table 2. The effect of myeloid leukaemia inhibitory factor (LIF: 1000 U ml⁻¹) on the rate of trophoblast outgrowth (stage 2 to stage 3)^a of mouse embryos *in vitro*

Treatment	n	Percentage outgrowth ^b		
		120 h	144 h	168 h
Control	66	0	47.0	62.1
LIF	74	13.5**	85.1**	85.1*

^aStage 2: trophoblast cells attached to bottom of culture dish; stage 3: trophoblast cells spread out on the bottom of culture dish.^bTime after hCG injection.

Asterisks indicate significant differences from respective control groups:

P* < 0.05; *P* < 0.01.**Table 3.** The effect of different culture periods in medium supplemented with myeloid leukaemia inhibitory factor (LIF: 1000 U ml⁻¹) on mouse embryo development after the blastocyst stage *in vitro*

Period of culture with LIF	n	Percentage developing to stage 3 ^a
None (control)	35	54.3
8-cell to stage 3	35	77.1
8-cell to blastocyst	35	65.7
Blastocyst to stage 3	35	85.7**

^aStage 3: trophoblast cells spread out on the bottom of the culture dish.**Significantly different from control (*P* < 0.01).

47% of the embryos cultured in unsupplemented medium (*P* < 0.01). An additional 24 h was required by these control embryos for all hatched blastocysts to reach stage 3 (Table 2). Thus LIF clearly advanced the rate of hatching and trophoblast outgrowth of eight-cell embryos *in vitro*.

Effects of LIF on development after the blastocyst stage

Eight-cell embryos were cultured *in vitro* in unsupplemented medium to the blastocyst stage, treated with LIF and analysed for developmental effects to determine the developmental stage at which embryos are responsive to LIF. The addition of 1000 U LIF ml⁻¹ resulted in a significant (*P* < 0.01) increase in the number of embryos reaching stage 3 (trophoblast outgrowth) compared with embryos cultured continuously in unsupplemented medium (85.7% or 30 of 35 versus 54.3% or 19 of 35). In contrast, addition of LIF to embryos between the eight-cell stage and the blastocyst stage failed to affect subsequent development. Blastocysts were derived from eight-cell embryos cultured in the presence or absence of 1000 U LIF ml⁻¹. Continued culture of these embryos in unsupplemented medium revealed no significant difference in the ability of these embryos to reach stage 3 of development (65.7% versus 54.3%) (Table 3). These results clearly indicate that mouse embryos

Fig. 1. (a) Four control mouse embryos (unsupplemented medium) and (b) two mouse embryos treated with myeloid leukaemia inhibitory factor cultured for 5 days *in vitro*. The arrows indicate the expanded trophoblast and the arrowheads indicate an inner cell mass. Scale bars represent 100 µm.

respond to LIF *in vitro* only after the eight-cell stage of development.

Effects of LIF on embryo inner cell mass and trophoblast

Image analyses were performed to measure the area of inner cell mass and the trophoblastic outgrowth of embryos. At day 5 of culture, the mean trophoblastic area of embryos cultured in LIF-supplemented medium (1000 U ml^{-1}) was 27% greater than controls ($111 \pm 30.7 \mu\text{m}^2$ versus $141 \pm 35.2 \mu\text{m}^2$, respectively; $P < 0.005$). There were no significant ($P < 0.05$) differences in the areas of the inner cell mass of embryos cultured in unsupplemented medium ($8.0 \pm 3.5 \mu\text{m}^2$) and LIF-treated embryos ($8.3 \pm 3.4 \mu\text{m}^2$) at this stage of culture (Fig. 1). By day 7, the mean area of the inner cell mass of LIF-treated embryos ($15.9 \pm 10.6 \mu\text{m}^2$) was significantly ($P < 0.025$) greater than that of the control embryos ($10.3 \pm 5.7 \mu\text{m}^2$), and the trophoblastic area was 43% greater ($P < 0.005$) in LIF-treated embryos ($296 \pm 67.2 \mu\text{m}^2$) than in embryos cultured in unsupplemented medium ($207 \pm 67.8 \mu\text{m}^2$). Thus LIF-treated embryos exhibit greater trophoblastic spreading as early as day 5 in culture.

Table 4. The effect of treating mouse embryos with myeloid leukaemia inhibitory factor (LIF: 1000 U ml^{-1}) during culture *in vitro* followed by embryo transfer to synchronized pseudo-pregnant females

Parameter	Stage of embryo at time of transfer			
	Hatching		Hatched	
	Control	LIF	Control	LIF
Number transferred	52	52	32	32
Percentage implanted	96.2	90.4	84.4	81.3
Percentage reabsorbed	30.0	29.8	33.3	19.2***
Percentage fetuses	70.0	70.2	66.7	80.8*

Asterisks indicate significant differences from respective control groups: * $P < 0.05$; *** $P < 0.001$.

Embryo viability following treatment with LIF

Eight-cell embryos were collected, cultured for 48–50 h with or without LIF and then transferred to the uteri of day 2.5 pseudopregnant recipient mice. Embryos were cultured until they developed to either the hatching stage of development, or until they had completely hatched *in vitro*. Only embryos from the unsupplemented medium group and the LIF-supplemented group that had attained each of these stages of development were then transferred to pseudopregnant females. On day 15 of gestation, the recipient mice were killed and their reproductive tracts were examined. The LIF-treated group that had been transferred at the hatching blastocyst stage exhibited no significant ($P > 0.05$) differences in development from those of the unsupplemented group (Table 4). However, there were fewer embryos reabsorbed in the LIF-treated group of embryos that were transferred at the hatched stage (19.2%) compared with controls (33.3%) ($P < 0.001$). Reabsorptions of untreated and LIF-treated embryos were randomly distributed among females. In concord with the fewer reabsorptions, a significant ($P < 0.05$) increase in the number of live fetuses was also observed in the LIF-treated group (80.8%) compared with the unsupplemented group (66.7%) (Table 4). Thus, LIF treatment increased the survival of embryos *in vivo*.

LIF expression in uterine cells *in vitro*

Expression of the LIF transcript (diffusible form), which in the ribonuclease protection assay is viewed as a protected band of 368 bp, was observed in endometrial cells cultured as monolayers from females at day 3 of pregnancy (Fig. 2). The expression of this transcript at relatively high concentrations is consistent with reports of LIF (diffusible form) expression by the uterus in pregnant and virgin females (Bhatt *et al.*, 1991; Shen and Leder 1991; Robertson *et al.*, 1993). Expression of the transcript was not significantly affected by treatment of the cells with progesterone ($3.2 \times 10^{-6} \text{ mol l}^{-1}$) or oestradiol ($3.7 \times 10^{-5} \text{ mol l}^{-1}$) alone or in combination.

Fig. 2. Expression of transcripts of myeloid leukaemia inhibitory factor (LIF) by uterine monolayers *in vitro*. RNase protection analysis of LIF transcripts (large arrow) was carried out as reported previously (Rathjen *et al.*, 1990a) using 5 µg total RNA isolated from: (lane 1) embryonic stem cells; endometrial cells from day 3 pregnant mouse uteri following culture with (lane 2) oestradiol (3.7×10^{-5} mol l $^{-1}$) and progesterone (3.2×10^{-6} mol l $^{-1}$), (lane 3) oestradiol (3.7×10^{-5} mol l $^{-1}$) alone, (lane 4) progesterone (3.2×10^{-6} mol l $^{-1}$) alone and (lane 5) without steroid hormones. Mouse glyceraldehyde-3-phosphate dehydrogenase (GAP) (small arrows) was used as a loading control. One representative experiment of two performed.

Discussion

In the present work, exogenous soluble LIF was shown to enhance the development of early mouse embryos *in vitro*. Treatment of embryos with LIF resulted in accelerated hatching, increased survival, increased proliferation of trophoblast cells and growth of the inner cell mass. Embryonic survival and progression *in vivo*, as evidenced by the transfer of embryos to pseudopregnant mice, was also enhanced by LIF treatment of embryos *in vitro*.

The action of LIF in promoting embryonic development was most effective at about the time of blastocyst formation. Treatment of embryos with LIF at earlier developmental stages, such as at the one-eight cell stage, when *in vivo* the embryo is located within the oviduct, had no measurable effect on embryogenesis *in vitro*. This lack of effect of LIF on embryos at the one-eight cell stage is interesting, given that the mouse oviduct is known to express LIF (Bhatt *et al.*, 1991; Shen and Leder, 1992) and it might be expected that the embryo at this stage would be bathed in LIF. The blastocyst stage of development in mice is followed by implantation of the embryo. At this stage there is a surge of LIF expression in the maternal endometrial glands and this appears to be necessary for blastocyst implantation (Stewart *et al.*, 1992). The principal effect of LIF on embryo development *in vitro* therefore coincides with the time at which maternal expression of the factor is expected to be highest.

The stimulatory effects of LIF on embryogenesis were manifest in greater inner cell mass and increased trophoblast proliferation. This increase in inner cell mass and trophoblast proliferation could represent a direct effect of LIF on these cells, given that LIF receptors have been identified on cells of the trophoblast (Fry, 1992) and on embryonic stem cells (Williams *et al.*, 1988) which are analogous to the cells of the inner cell mass. Biological actions of LIF on trophoblast cells have not been described. LIF prevents the differentiation of embryonic stem cells *in vivo* (Smith *et al.*, 1988; Williams *et al.*, 1988), which in an embryonic context could result in increased proliferation of the inner cell mass. However, it is not known whether exogenous LIF can gain access to cells of the inner cell mass *in vitro*, although translocation of LIF across polar trophoblast in a manner analogous to that described for insulin (Heyner *et al.*, 1989) cannot be excluded. It is possible that all cells of the early embryo could be in contact with LIF *in vivo*, which is expressed at a variety of sites including the uterine lining and the extraembryonic portion of the conceptus. Although the embryotrophic effects of LIF could result from direct action on the inner cell mass and trophoblast cell populations, the possibility of secondary or paracrine interactions, initiated by exogenous LIF and resulting in embryotrophic effects, cannot be excluded.

Maternal expression of LIF in the uterus appears to be stimulated, directly or indirectly, by progesterone and oestradiol. These hormones enhance embryonic development in mice *in vitro* but only when early embryos are cultured in the presence of uterine monolayers; an effect which could be explained if the action of the hormones was indirect, perhaps via an embryotrophic factor produced by the uterine monolayer. LIF is a good candidate for such a factor given its known expression pattern *in vivo* and the embryotrophic effects reported here. Consistent with the known expression of LIF in the female reproductive tract, strong expression of the LIF (diffusible form) transcript by uterine monolayers *in vitro* was observed, suggesting the production of diffusible LIF. However, this LIF expression was not noticeably increased by the addition of progesterone and oestradiol, alone or in combination. The failure to observe a steroid-mediated increase of LIF transcription in these cells could reflect a requirement for an intermediate factor not present in this system, or possibly that LIF transcription can be greatly increased *in vivo* by factors often present in serum (Rathjen *et al.*, 1990a).

The observation that uterine monolayers express LIF transcripts *in vitro* and that LIF can have an embryotrophic effect on early development provide an explanation for the previously reported stimulatory effects of uterine monolayers on early development in mice *in vitro* (Lavranos and Seamark, 1989). This earlier study on endometrial cell and embryo coculture showed no enhancement of embryo development when eight-cell embryos were cultured with steroid hormones alone or with uterine cells alone. The enhancement of embryonic growth and development by steroid hormones was seen only in coculture. The enhanced embryotrophic effect induced in this coculture system by steroid hormones would, however, need to be explained by an alternative hypothesis, since treatment of the uterine monolayers with steroid hormones did not alter LIF expression. The positive effects of steroid hormones on embryogenesis in mice in the presence of uterine monolayers

must therefore be distinct from the embryotrophic effects of exogenous LIF reported in this paper. This implicates the secretion of other embryotrophic factors by uterine monolayers in response to steroid hormones, or the reorganization of the uterine epithelium to provide the embryo with an improved environmental matrix. In this regard it is interesting that other cytokines and growth factors, including CSF-1 (Arceci *et al.*, 1989), can be produced by the uterine epithelium. The relationship between the LIF-mediated and steroid-mediated effects on embryogenesis *in vitro*, and the known requirement for LIF for implantation *in vivo*, cannot be assessed on the basis of the data presented here.

The data presented in this paper, in combination with previous reports, indicate that LIF may have a dual role in early development in mice. Maternal LIF production is necessary for embryo implantation. It is possible that LIF may have a secondary role, as an embryotrophic factor. Data obtained from gene inactivation in mice indicate that there is no absolute requirement for embryonic LIF expression for the development of the embryo, although the observation that LIF-deficient embryos are smaller than heterozygous littermates (Stewart *et al.*, 1992) is interesting given our identification of the embryotrophic activity of LIF. Maternal LIF expression is not required for development of the embryo to the blastocyst stage, since viable blastocysts can be recovered from LIF-deficient mothers (Stewart *et al.*, 1992). Potential embryotrophic effects of maternal LIF expression beyond this point of embryogenesis cannot be easily assessed by gene inactivation strategies which result in the absence of embryo implantation. This is unfortunate given that our results indicate that the main embryotrophic effects of LIF follow formation of the blastocyst, and possibly implantation.

Interactions between mother and fetus during early embryogenesis are complex but critical for regulated construction of the embryonic environment. The importance of maternal LIF expression in this process has been established by mutational analyses. We have now demonstrated a potential second role for LIF in early embryogenesis, as an embryotrophin. Our suggestion that there may be multiple roles for LIF at this stage of development is compatible with the multiple embryonic and extraembryonic expression sites, and indicates the complexity of the regulatory network governing maternal-fetal interactions.

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