

Leukaemia inhibitory factor expression in human follicular fluid and ovarian cells

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Leukaemia inhibitory factor (LIF) is a 43 kDa glycoprotein with a remarkable range of biological actions in different tissue systems. LIF improves the rate of fertilization of mouse oocytes *in vitro* and up-regulates aromatase enzyme. We postulated that LIF may be an important modulator of ovarian function and may also improve embryo quality in humans. Follicular fluid samples from patients undergoing in-vitro fertilization (IVF) and embryo transfer ($n = 123$), from women undergoing ovarian stimulation ($n = 4$) and from women undergoing laparoscopy for tubal ligation during their follicular phase ($n = 3$) were used. Follicular fluid LIF, oestradiol, and progesterone were measured and embryo quality was assessed. Granulosa-lutein cells were cultured for 3 days in Ham's F-12:Dulbecco's modified Eagle's medium (DMEM). Ovarian stromal cells, isolated by enzymatic dispersion of ovarian tissue, were also cultured in the same medium. Following experimental treatments, LIF mRNA and protein concentrations were quantified. The concentration of LIF was 0.8 ± 0.3 (mean \pm SEM) pg/ml in pre-human chorionic gonadotrophin (HCG) follicular fluid samples and 13.0 ± 1.1 pg/ml in post-HCG follicular fluid samples ($P < 0.05$). LIF levels were undetectable in three follicular fluid samples obtained during unstimulated follicular phase. There was a correlation between follicular fluid LIF and follicular fluid oestradiol concentrations ($r = 0.36$; $P = 0.0001$) and the number of grade I embryos ($r = 0.62$; $P = 0.01$). LIF mRNA and the protein were expressed constitutively but in low amounts in the ovarian stromal cell cultures. The concentrations of LIF mRNA as well as protein were increased by interleukin (IL)-1 α and tumour necrosis factor α (TNF α) in a time- and concentration-dependent manner. Purified granulosa-lutein cells expressed low amounts of LIF mRNA and protein which were not significantly increased by IL-1 α or TNF α . Our findings suggest that HCG stimulates the expression of LIF in follicular fluid. Both granulosa-lutein and ovarian stromal cells express the LIF mRNA and produce the protein. Modulation of LIF in these cells may play an important role in the physiology of ovulation and early embryo development.

Key words: follicular fluid/granulosa-lutein cells/leukaemia inhibitory factor/ovary/tumour necrosis factor α

Introduction

The micro-environment of human follicles is vital for normal oocyte development, folliculogenesis, and timely ovulation. Numerous studies have shown that a variety of cytokines are capable of affecting ovarian function and are implicated as regulators of gonadal steroid secretion, corpus luteum function, embryo development, and implantation (Brannstrom and Norman, 1993; Adashi, 1994). Follicular fluid provides the environment in which oocyte maturation occurs, and in turn should affect fertilization and early embryonic development. In assisted reproductive technologies, follicular fluid has been shown to improve the in-vitro development of human pre-embryos and pregnancy rates (Fakih and Vijayakumar, 1990; Hemmings *et al.*, 1994).

Leukaemia inhibitory factor (LIF) is a 43 kDa glycoprotein with a remarkable range of biological actions in different tissue systems. LIF regulates the growth and differentiation of embryonic stem cells (Williams *et al.*, 1988; Smith *et al.*, 1992), primordial germ cells (Matsui *et al.*, 1991), peripheral neurons (Yamamori *et al.*, 1989; Murphy *et al.*, 1991), osteoblasts (Reid *et al.*, 1990), adipocytes (Mori *et al.*, 1989), hepatocytes (Baumann and Wong, 1989) and endothelial cells (Arai *et al.*, 1990). LIF also improves the mouse blastocyst development *in vitro* (Kauma and Matt, 1995). Early murine embryos cultured with LIF demonstrate increased trophoblast mass and an increased rate of hatching (Lavranos and Seamark, 1989; Fry, 1992). In ovine embryo experimentation, the addition of human LIF to in-vitro culture media increased the number of blastomeres, decreased the rate of embryo degeneration, increased the rate of blastocyst hatching and increased the pregnancy rate (Fry *et al.*, 1992). Recently, LIF has also been shown significantly to enhance the blastocyst formation rates of human embryos (Dunlison *et al.*, 1996), a finding that remains controversial (Jurisicova *et al.*, 1995).

We postulated that LIF may be one of the factors in follicular fluid that improves early reproductive events. LIF was recently shown to increase the expression of aromatase in adipose tissue fibroblasts (Zhao *et al.*, 1995), and thus may affect oestrogen biosynthesis. Based on these findings, we also postulated that LIF may be an important modulator of ovarian function. In this regard, we investigated the expression and modulation of LIF in human follicular fluid samples and ovarian stromal and granulosa-lutein cell cultures.

Materials and methods

Follicular fluid and tissue collection

Follicular fluids and cells for granulosa-lutein cell culture were obtained from 123 patients (aged 24–43 years) undergoing in-vitro fertilization (IVF)-embryo transfer therapy at Yale University School of Medicine, USA. Written informed consent was obtained from each woman before the procedure; consent forms and protocols were approved by the human investigation committee of this university. Aetiological factors for infertility included 37% tubal factor, 33% male factor, 11% endometriosis, and 19% unexplained infertility. In addition 15 donor oocyte cycles without male factor were evaluated. A standard IVF protocol was used. Briefly, gonadotrophin-releasing hormone (GnRH)-agonist (leuprolide acetate, Lupron®; Tap Pharmaceuticals, Deerfield, IL, USA) was administered 1 mg per day s.c., starting in the midluteal phase of the preceding cycle or in 3% of cases on the first day of the stimulation cycle. Stimulation with human menopausal gonadotrophin (HMG) (Pergonal®; Serono Laboratories, Norwell, MA, USA) or follicle-stimulating hormone (FSH) (Metrodin®; Serono Laboratories) was initiated when there was no sonographic evidence of ovarian follicular activity and the serum oestradiol concentration was <50 pg/ml (conversion factor to SI unit, 3.671); and was continued until oestradiol concentrations reached 500 pg/ml or greater and at least two follicles of 17 mm or larger in diameter were present. At that time 10 000 mIU (HCG, Profasi®; Serono Laboratories) was administered and Lupron® and HMG were discontinued. Oocyte retrieval by transvaginal ultrasound guidance was performed at ~34 h after HCG administration. After removal of the cumulus-oocyte complexes, follicular fluids from a single patient were pooled then centrifuged at 600 g for 20 min. Grossly bloody samples were discarded. The cell-free supernatants were then aliquoted into polypropylene microfuge tubes and stored at –80°C until assayed. Cell pellets were used for culture of granulosa-lutein cells. Embryos were graded (I to IV) on the transfer day according to their morphology under the dissecting microscope according to Veck (1991). Type I embryos were the best and were defined as round and well shaped blastomeres without fragments. The four samples of follicular fluid collected before HCG administration were obtained from King Faisal Specialist Hospital and Research Centre, Riyadh, Kingdom of Saudi Arabia and shipped on dry ice and stored at –80°C until assayed together with other samples. Consent forms and protocols were approved by the human investigation committee of that institution. These follicular fluid samples were obtained from patients undergoing ovarian stimulation with GnRH-agonist and HMG for timed intra-uterine insemination. Patients underwent ultrasound guided follicular reduction (of follicles of 16 mm or larger in diameter) just prior to HCG administration to prevent ovarian hyperstimulation syndrome and/or multiple gestation. Additionally, three samples of follicular fluid were collected at Yale University School of Medicine from three women undergoing laparoscopy for tubal ligation during their follicular phase.

Ovarian tissue was obtained from women of reproductive age undergoing hysterectomy with oophorectomy for reasons other than ovarian disease ($n = 27$). Informed consent for the use of these tissues was obtained in writing from each woman prior to surgery. The consent forms and protocols used were approved by the human investigation committee of this university. The ovarian tissues were placed in culture media and transported to the laboratory for dissection of the visible follicles and culture of ovarian stromal cells.

Isolation and culture of granulosa-lutein cells

Cell pellets obtained after the centrifugation of follicular fluids were resuspended in Hanks' balanced salt solution (HBSS). The cell

suspension was gently layered over Ficoll (LSM, Organon Teknika, Durham, NC, USA) and centrifuged at 500 g for 20 min to pellet red blood cells. Cells at the interface were removed with a Pasteur pipette, resuspended in HBSS, and centrifuged at 500 g for 10 min. Finally, the cell pellet was resuspended in Ham's F-12/Dulbecco's minimal essential medium (DMEM, 1:1, v/v) that contained antibiotics-antimycotics (1%, v/v) and fetal bovine serum (FBS, 10%, v/v). The average viability was 85% as assessed by a dye exclusion test using trypan blue (Gibco BRL, Grand Island, NY, USA). Dispersed cells were counted using a haemocytometer; cell number varied from 10×10^6 to 30×10^6 cells per patient. Cell suspensions were then diluted accordingly to a concentration of 5×10^5 viable cells/ml and plated in 6-well plates. The plates were incubated for 1–6 days at 37°C in a humidified atmosphere of 5% CO₂ in air with daily replacement of medium.

In some experiments, granulosa-lutein cells were treated with the monoclonal anti-CD45 antibody coupled with magnetic immunobeads (Amac, Westbrook, ME, USA) to remove white blood cells. Isolated granulosa-lutein cells were suspended in 2 ml of medium that contained 30% fetal calf serum and incubated for 10 min at room temperature with anti-CD45 immunomagnetic beads. The suspension was then placed into a magnetic test tube rack (Bio-Mag, Lexington, MA, USA) for 10 min at room temperature, resulting in the removal of immunobead-bound white blood cells from the cell suspension. After careful removal of the supernatant, the process was repeated twice under identical conditions and 'purified' granulosa-lutein cells were centrifuged and resuspended in Ham's F-12/DMEM. The viability of cells was 95% as assessed by the dye exclusion test using trypan blue. The granulosa-lutein cells were plated in 24-well plates at 5×10^4 cells/well density for experiments.

At the end of each experiment, the culture media were collected and frozen at –80°C for quantification of LIF by enzyme-linked immunosorbent assay (ELISA). Cells were used for quantification of total protein or for isolation of RNA. Similar experiments were conducted on at least three different occasions.

Isolation and culture of ovarian stromal cells

Ovarian tissue cleared from visible follicles was digested by incubation of tissue minces in HBSS that contained HEPES (25 mM), penicillin (200 U/ml), streptomycin (200 mg/ml), collagenase (1 mg/ml, 15 U/mg), and DNase (0.1 mg/ml, 1500 U/mg) for 2 h at 37°C with agitation. The dispersed cells were filtered through a wire sieve (73 µm diameter pore) to remove undigested tissue pieces. The ovarian stromal cells were plated in Ham's F12/DMEM (1:1, v/v) that contained antibiotics-antimycotics (1%, v/v) and FBS (10%, v/v). Cells were plated in plastic flasks (75 cm²), maintained at 37°C in a humidified atmosphere (5% CO₂ in air), and allowed to replicate to confluence. Thereafter, the stromal cells were passed by standard methods of trypsinization and plated in culture dishes (100 mm diameter or 24-well plates) as appropriate for the experimental design, and allowed to replicate to confluence. Experiments were commenced 1–3 days after confluence was attained. Because we have previously shown in other cell types that serum stimulates LIF production (Arici *et al.*, 1995), the confluent cells were treated with serum-free media for 24 h before treatment with test agents was initiated. For cytokine modulation experiments, confluent cells were treated with human recombinant cytokines (IL-1α and TNFα) dissolved in phosphate-buffered saline containing 0.01% bovine serum albumin as carrier protein. Control cells were treated with vehicle only (phosphate-buffered saline containing 0.01% bovine serum albumin).

At the end of each experiment, the culture media were collected and frozen at –80°C for quantification of LIF by ELISA. Cells were used for quantification of total protein or for isolation of RNA.

Similar experiments were conducted on at least three different occasions.

Immunocytochemical analyses of granulosa-lutein and ovarian stromal cells

Immunocytochemical analyses of granulosa-lutein and ovarian stromal cells were conducted using factor VIII (Dako, Carpinteria, CA, USA) as a marker of endothelial cells, low molecular weight cytokeratin (Dako) as a marker of epithelial cells, vimentin (BioGenex, San Ramon, CA, USA) as a marker of mesenchymal cells, aromatase (generously donated by Dr N. Harada, Fujida Health University, Toyoake, Aichi, Japan) as a marker of oestrogen producing cells, and HAM 56 (Dako) as a marker of monocyte/macrophages (Horny *et al.*, 1994). Freshly isolated granulosa-lutein cells and cultured ovarian stromal cells at first passage were plated on 22 mm² cover slips in 35 mm wells. Cells were fixed in 4% paraformaldehyde and stored at -20°C. Cells were exposed to monoclonal murine anti-human antibodies (for factor VIII, 1:100 dilution) after a permeabilization step; for low molecular weight cytokeratin (1:500 dilution); for vimentin (1:30 dilution); for HAM 56 (1:150 dilution); for aromatase (1:2000 dilution). An avidin-biotin developing system (Vectostain ABC kit[®], Vector Labs, Burlingame, CA, USA) was used, as described.

In the granulosa-lutein cell cultures, macrophage-marker-positive cells, endothelial cells, and cytokeratin-positive cells accounted for 6–9%, 1–2%, and 0% respectively. Nearly all of the cells (95 to 100%) were positive for vimentin. Macrophage-marker-positive cells represented less than 1% after treatment with anti-CD45 immunobeads. In confluent ovarian stromal cell cultures after first passage, macrophage-marker-positive cells, endothelial cells, and cytokeratin-positive cells accounted for 2–4%, 1–2%, and 5–7% respectively. Nearly all the cells (95–100%) were positive for vimentin. Approximately 10–15% of the cells were positive for aromatase. Because of the low percentage of leukocytes in stromal cell culture, CD45 depletion was not performed in these cells. These data were compiled from preparations of cells from three different samples.

Preparation of total RNA and Northern analysis

Total RNA from cells in culture was extracted using Trizol[®] (Gibco BRL). Total RNA was size-fractionated by electrophoresis on 1% formaldehyde-agarose gels, transferred electrophoretically to Hybond-N⁺ membrane (Amersham, Arlington Heights, IL, USA), and cross-linked to the membrane by use of UV light. Prehybridization was conducted for 5 h at 42°C in buffer comprised of 5× SSC, 5× Denhardt solution, formamide (50%, v/v), dextran sulphate (5%, w/v), NaH₂PO₄ (50 mM) and salmon sperm DNA (0.5 mg/ml). Hybridizations were conducted for 16 h at 42°C in buffer composed of 5× SSC, 2× Denhardt solution, formamide (50%, v/v), dextran sulphate (10%, w/v), NaH₂PO₄ (20 mM), and salmon sperm DNA (0.1 mg/ml) with a cDNA probe (5–15 µCi) complementary to LIF mRNA radiolabelled with [α -³²P]dCTP by random hexamer priming. The human LIF cDNA (pXM.6R) used in this study was kindly provided to us by Genetics Institute Inc. (Cambridge, MA, USA) and is complementary to full length LIF mRNA (Moreau *et al.*, 1988; Denhez *et al.*, 1990). After hybridizing, the blots were washed with 1× SSC and SDS (0.1%, w/v) for 15 min at room temperature, once with 0.1× SSC and SDS (0.1%, w/v) for 15 min at room temperature, and once for 20 min at 65°C. Autoradiography of the membranes was performed at -70°C using Kodak X-Omat AR film. The presence of equal amounts of total RNA in each lane was verified by visualization of ethidium bromide-stained 28S and 18S ribosomal RNA subunits and by analysis of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA, using a cDNA probe (Clontech

Laboratories, Palo Alto, CA, USA) radiolabelled with [α -³²P]dCTP by random hexamer priming. The autoradiographic bands were quantified by using a laser densitometer (Molecular Dynamics, Sunnyvale, CA, USA). Each LIF band was normalized by using the value for the corresponding G3PDH mRNA, thus correcting for any variation in amounts of RNA applied to each lane. Similar experiments were conducted on three different occasions with cells prepared from different patients.

LIF immunoassay

Immunoreactive LIF in follicular fluid samples and culture supernatant was quantified using an ELISA from R&D Systems (Minneapolis, MN, USA). According to the manufacturer, there is no measurable cross-reactivity with other known cytokines in this assay. The sensitivity for LIF was 2 pg/ml. Follicular fluid samples were evaluated in duplicate assay. Validation of its use for human follicular fluid was also performed: recombinant LIF was diluted in assay buffer and pooled follicular fluid and parallelism was observed between the standard curve of buffer and follicular fluid dilutions. Each experiment was done using three replicate wells for each condition and supernatant from each well was tested in a single ELISA assay. Each experimental set-up was repeated on three or more occasions using cells obtained from three different patients. The intra-assay and interassay coefficients of variation were 3.6 and 5.4% respectively at 31.3 pg/ml concentration and were 8.9 and 11.8% respectively at 7.8 pg/ml concentration.

Progesterone and oestradiol immunoassays

Immunoreactive progesterone in follicular fluid and serum was quantified by radioimmunoassay (Progesterone MAIA, Biodata Diagnostics, Rome, Italy). According to the manufacturer, there is <1% cross-reactivity with other steroid hormones, the sensitivity for progesterone is 0.022 ng/ml and the intra-assay and interassay coefficients of variation are 8.06 and 7.71% respectively.

Immunoreactive oestradiol in follicular fluid and serum was quantified by radioimmunoassay (Estradiol MAIA, Biodata, Rome, Italy). According to the manufacturer, there is <1.8% cross-reactivity to other steroid hormones, the sensitivity for oestradiol is 5 pg/ml and the intra-assay and interassay coefficients of variation are 7.17 and 9.85% respectively. Due to high concentrations of steroids in follicular fluid, the samples were diluted 1:500 in steroid-free zero standard solution as recommended by the manufacturer.

Statistical analyses

Because the concentrations of LIF in follicular fluid were not normally distributed, they were analysed with non-parametric analysis of variance by ranks (Kruskal–Wallis). Individual groups were compared *post hoc* with the non-parametric Mann–Whitney test. Data from the ELISA assays were evaluated by analysis of variance with Bonferroni *post-hoc* analysis for multiple comparisons. Correlation analyses were performed using Pearson or Spearman rank tests as appropriate. Statistical calculations were performed using the Statistical Package for Social Sciences (SPSS) Version 6.0 for Windows (SPSS, Chicago, IL, USA).

Results

Immunoreactive LIF in follicular fluid

The mean concentration of LIF in follicular fluid samples obtained from women ($n = 4$) prior to the administration of HCG and prior to detectable LH surge was 0.8 ± 0.3 (mean \pm SEM) pg/ml. In follicular samples obtained from women

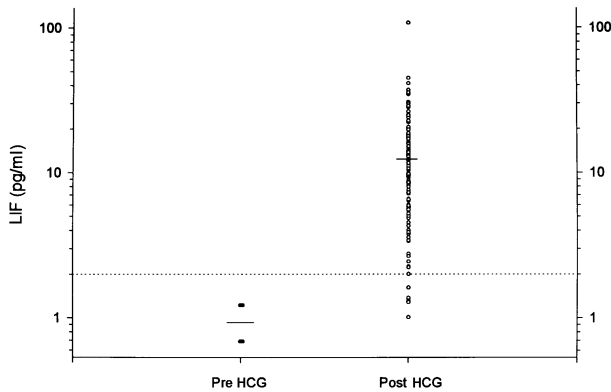


Figure 1. Distribution of immunoreactive leukaemia inhibitory factor (LIF) concentrations in follicular fluids obtained before ($n = 4$) or after ($n = 123$) human chorionic gonadotrophin (HCG) administration. Horizontal lines represent the medians, dashed line represents lowest detection limit of the enzyme-linked immunosorbent assay (2 pg/ml). $P < 0.05$ for pre-HCG versus post-HCG groups.

($n = 123$) 34 h after the HCG administration the mean concentration of LIF was 13.0 ± 1.1 pg/ml which was significantly higher than pre-HCG levels ($P < 0.05$) (Figure 1). The mean diameter of aspirated follicles was 17 mm (range 15–22). In three samples of follicular fluid obtained from three women undergoing laparoscopy for tubal ligation during their follicular phase prior to a detectable LH surge (cycle days 8, 12 and 14; follicular diameter 12, 16 and 18 mm respectively), LIF concentrations were undetectable.

In follicular samples where oestradiol concentrations were measured ($n = 102$), there was a correlation between follicular fluid LIF and follicular fluid oestradiol concentrations from individual patient samples ($r = 0.36$; $P = 0.0001$). No correlation was observed between follicular fluid LIF concentrations and follicular fluid progesterone, serum oestradiol, or serum progesterone concentrations.

We then evaluated the correlation between follicular fluid LIF concentrations and embryo quality in cycles of oocyte donors ($n = 15$). This subgroup of patients was selected in order to decrease variability that may be introduced by other confounding factors such as maternal age or male factor. There was a correlation between follicular fluid LIF concentrations and the number of grade I embryos ($r = 0.62$; $P = 0.01$) (Figure 2A). There was also a correlation between follicular fluid LIF concentrations and average embryo grade ($r = -0.51$; $P < 0.05$) in the same group (Figure 2B). In the remaining group of patients (with male or tubal factor, or unexplained infertility) there was no significant correlation between follicular fluid LIF concentrations and embryo quality. A weak correlation between follicular fluid LIF concentrations and average oocyte grade in the male infertility group was observed ($r = 0.38$; $P = 0.06$). In the remaining groups of patients there was no significant correlation between follicular fluid LIF concentrations and oocyte grade.

Cytokine modulation of LIF production and mRNA expression in granulosa-lutein and ovarian stromal cells

All results are from representative experiments performed on at least three occasions using cells originating from different

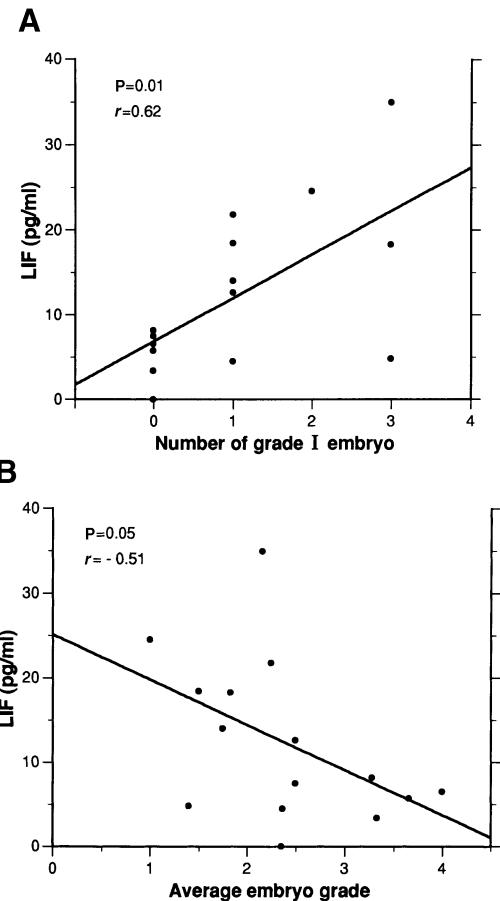


Figure 2. Correlation between follicular fluid leukaemia inhibitory factor (LIF) concentrations and embryo quality. LIF concentration was measured in follicular fluids of women undergoing ovarian stimulation for oocyte donation ($n = 15$) and plotted against the number of grade I embryos (A) and against the average embryo grade (B) for each woman.

individuals. Differences in absolute values between experiments did not affect observed relative increases and decreases in concentration. LIF mRNA was detectable in granulosa-lutein cells before elimination of leukocytes and increased in response to interleukin (IL)-1 α and tumour necrosis factor α (TNF α) treatment. In cultures performed after elimination of leukocytic cells by use of anti-CD45 immunobeads, granulosa-lutein cells secreted low concentrations of LIF into the culture medium (3.6 ± 0.8 pg/ml, equivalent to 84 ± 9 pg/mg of total protein by 8 h). Treatment of granulosa-lutein cells with IL-1 α (10–100 U/ml) and TNF α (1–10 ng/ml) alone or in combination did not result in a significant increase in LIF production (data not shown).

Ovarian stromal cells secreted low concentrations of LIF (4 ± 2 pg/ml, equivalent to 28 ± 14 pg/mg of total protein by 8 h). Treatment of ovarian stromal cells with IL-1 α (10 U/ml) resulted in increased accumulation of LIF in the media (28 ± 6 pg/ml, equivalent to 205 ± 40 pg/mg of total protein by 8 h) ($P = 0.01$). Similar findings were also observed following treatment with TNF α (10 ng/ml) by 8 h (35 ± 21 pg/ml, equivalent to 247 ± 124 pg/mg of total protein by 8 h). Treatment of ovarian stromal cells with IL-1 α (10 U/ml) and TNF α (10 ng/ml) in combination resulted

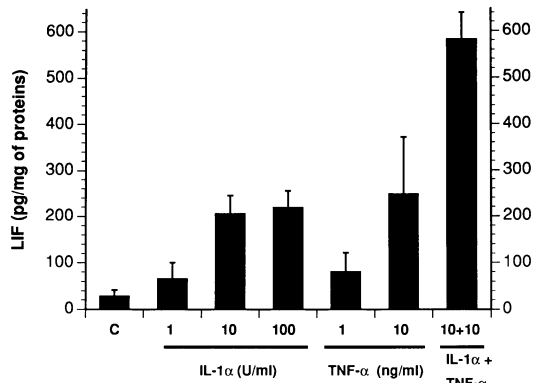


Figure 3. Stimulation of immunoreactive leukaemia inhibitory factor (LIF) production by ovarian stromal cells in culture by interleukin (IL)-1 α and tumour necrosis factor α (TNF α). Confluent ovarian stromal cells were treated with medium alone (control) or with medium containing IL-1 α (1–100 U/ml) or TNF α (1–10 ng/ml) or both for 8 h. The culture media were collected, and LIF was quantified by enzyme-linked immunosorbent assay. Data are mean \pm SEM for three replicates.

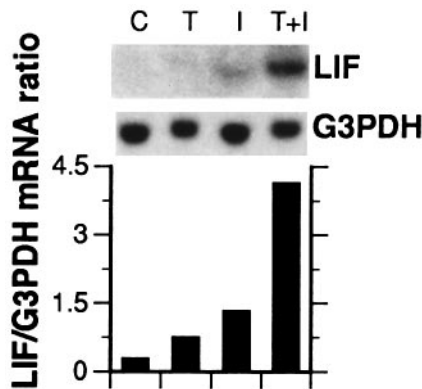


Figure 4. Northern analysis of leukaemia inhibitory factor (LIF) mRNA in ovarian stromal cells treated with interleukin (IL)-1 α and tumour necrosis factor α (TNF α). Confluent ovarian stromal cells were incubated for 6 h with IL-1 α (10 U/ml) or TNF α (10 ng/ml) or both. Total RNA (10 μ g per lane) was evaluated. C: control; I: IL-1 α ; T: TNF α . G3PDH = glyceraldehyde-3-phosphate dehydrogenase.

in an additive increased accumulation of LIF in the media (62 ± 7 pg/ml, equivalent to 512 ± 79 pg/mg of total protein by 8 h) ($P = 0.01$) (Figure 3). LIF mRNA was low but detectable in stromal cells maintained in serum-free medium. The amount of LIF mRNA increased markedly in response to IL-1 α and TNF α treatment alone or in combination (Figure 4). This increase was evident by 15 min (data not shown), and high concentrations of LIF mRNA were observed after 2–4 h of either treatment before beginning to decrease (Figure 5, Panel A and B). The increase in LIF mRNA in stromal cells was dependent upon the concentration of IL-1 α (0.01–10 U/ml) or TNF α (0.01 to 1 ng/ml) (data not shown).

Discussion

Successful ovulation requires an integrated series of events that ultimately leads to the timely release of a mature oocyte from the follicle. The LH surge initiates the final maturation

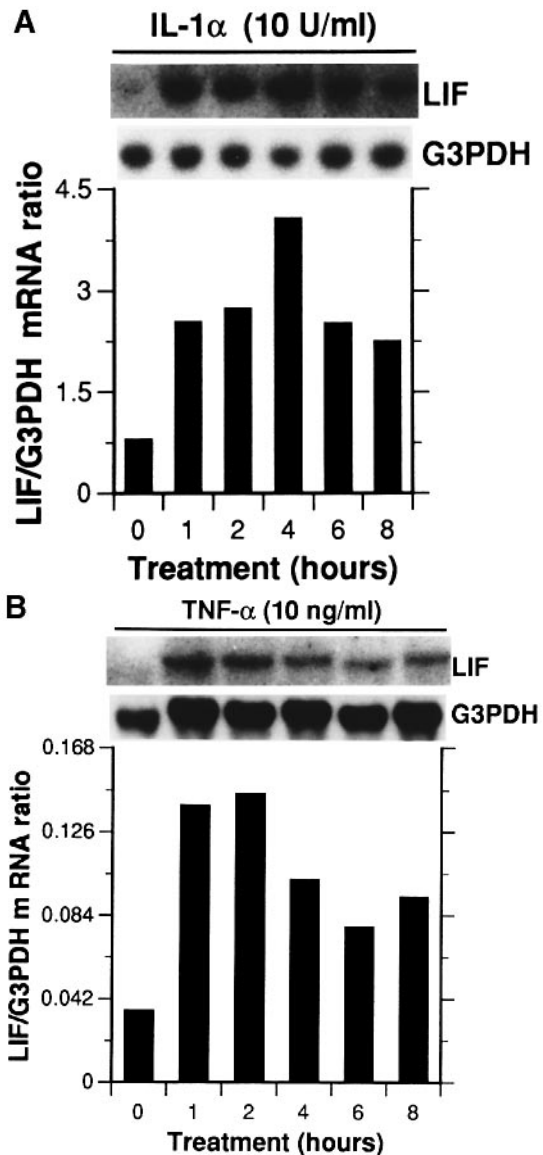


Figure 5. Interleukin (IL)-1 α and tumour necrosis factor α (TNF α)-mediated increases in leukaemia inhibitory factor (LIF) mRNA concentrations. Confluent ovarian stromal cells were placed in serum-free medium 24 h before incubation with culture medium containing IL-1 α (10 U/ml) for 1–12 h (A) or TNF α (10 ng/ml) for 1–8 h (B). At the end of the incubation period, the culture media were removed, and total RNA was prepared from the cells. LIF mRNA was evaluated by Northern analysis of total RNA (10 μ g per lane). G3PDH = glyceraldehyde-3-phosphate dehydrogenase.

of the oocyte and simultaneously triggers the cascade of events leading to follicular rupture and luteinization of granulosa cells. If LIF is truly involved in the peri-ovulatory events, the concentration of LIF should be increased in follicular fluids obtained after the LH surge or the administration of HCG versus follicular fluids obtained before these events. This is precisely the finding of this study: we found LIF in greater quantities in follicles after HCG administration than before. We investigated the expression and modulation of LIF in the human ovarian follicle because of the demonstrated importance of LIF in supporting successful early embryonic development (Fry, 1992). We further speculated that treatment with cytokines

that were shown to enhance LIF expression in the endometrium (Arici *et al.*, 1995) might similarly modulate LIF in ovarian cells.

Follicular fluid provides an embryotrophic environment which can improve development of human pre-embryos and pregnancy rates (Fakih and Vijayakumar, 1990; Hemmings *et al.*, 1994). We expected that follicular fluid LIF concentrations would be correlated with embryo quality. We observed significant correlation between embryo quality and corresponding follicular fluid LIF concentrations among embryos from donated oocyte cycles. Since LIF was shown to increase the expression of aromatase in adipose tissue fibroblasts (Zhao *et al.*, 1995), we also expected and found a correlation between follicular fluid LIF concentrations and follicular fluid oestradiol concentrations. Thus, LIF may be one of the factors affecting oestrogen biosynthesis in the ovarian follicle. On the other hand, our study does not prove a cause and effect relationship between LIF and either intrafollicular oestradiol production or embryo quality. There is always a possibility that all three events are independently induced by another, yet-to-be-determined causative agent. Initial studies by Stewart *et al.* (1992) revealed that ovarian function in transgenic mice lacking LIF was not affected and these animals could produce normal embryos, but the embryos failed to implant.

Although granulosa-lutein cells predominate in follicular fluid, resident macrophages and monocytes comprise 5–15% of human follicular tissue cells (Loukides *et al.*, 1990) and these cells are a known source of LIF. We found that after purification of granulosa-lutein cells by use of anti-CD45 immunobeads, the production of LIF in response to IL-1 and TNF α decreased markedly. Thus, it appears that macrophages are also contributing to the LIF pool. The other potential source of LIF is the theca cells surrounding the follicles. We found that cultured ovarian stromal cells express LIF mRNA and secrete LIF protein. Thus, sources of LIF are abundant in and around the follicle. Peripheral blood contamination is always a potential source during the process of aspiration, but the LIF concentrations observed in follicular fluid are substantially higher than those observed in serum (mostly undetectable). Therefore, the markedly elevated LIF concentrations should reflect the true follicular fluid concentrations and should not be secondary to contamination by peripheral blood. The bulk of our data is obtained from analysis of follicular fluids obtained from women undergoing stimulation cycles and may not represent a physiological process. On the other hand, in three follicular fluid samples obtained from women during unstimulated follicular phase and in four samples obtained during stimulated cycles, all obtained prior to detectable LH surge, LIF concentrations were below the detection limit of LIF assay.

The final question we asked was how LIF secretion might be regulated. This study presents evidence that the expression of this cytokine from ovarian stromal cells is modulated by other cytokines such as IL-1 and TNF α . The complete intra-ovarian IL-1 system with ligands, receptors, and a receptor antagonist is now well established in humans (Hurwitz *et al.*, 1992). TNF α is also found in the pre-ovulatory follicular fluid

at concentrations used in the present study (Wang *et al.*, 1992). Thus, both cytokines may play some role in the secretion of LIF.

In summary, we find that LIF concentrations are elevated in peri-ovulatory follicular fluid and both granulosa-lutein cells and ovarian stromal cells express LIF mRNA and produce the protein. Modulation of LIF in these cells suggests that LIF plays an important role in the physiology of ovulation, oestrogen production and early embryonic development.

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