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IN VITRO ACTION OF LEUKEMIA INHIBITORY FACTOR (LIF) ON MID-SECRETORY STAGE ENDOMETRIAL STROMAL CELLS COLLECTED FROM HORMONE-SIMULATED, OVARIECTOMIZED MONKEY AND MAINTAINED IN THREE-DIMENSIONAL PRIMARY CULTURE

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Abstract : Leukemia inhibitory factor (LIF) is a pleiotropic cytokine that is known to play an important role in blastocyst implantation. The putative action of LIF in the regulation of uterine function has been examined using mid-secretory stage monkey endometrial stromal cells cultured on rat-tail collagen type I and treated with recombinant human LIF (rhLIF) or immunoneutralized LIF (in LIF) under serum-free condition. Long-term ovariectomized rhesus monkeys (n=8) underwent simulation of their menstrual cycles with steroid hormones and endometrial tissue samples were collected on cycle day 18; stromal cells were isolated and grown in primary culture on three-dimensional collagen matrix. Significant decline in cellular protein synthesis ($P<0.01$) and cell proliferation index ($P<0.05$) was observed in cells with increasing doses (0–1000 ng/ml) of rhLIF under serum-free *in vitro* condition. JAK1 expression in cultured cells increased ($P<0.01$) in response to rhLIF as revealed from Western blot and confocal laser scanning microscopic examination, STAT1 and STAT2 expressions were unchanged, while pSTAT3 expression increased ($P<0.01$) with increased concentration of rhLIF in culture medium. Autophosphorylation of JAK1 in endometrial stromal cells showed no change with increasing concentration (0.01 to 100 ng/ml) of rhLIF *in vitro*, but significant ($P<0.05$) increase was observed with the time of exposure to rhLIF. Immunoneutralization of LIF or no addition of rhLIF to cultured cells led to significant ($P<0.01$) increase in stromal cell proliferation index and significant ($P<0.01$) decrease in the level of JAK1 and its autophosphorylation as compared to cells exposed to rhLIF alone. From the present set of experiments we conclude that rhLIF affects the physiological behaviour of monkey mid secretory stage endometrial stromal cells *in vitro* via the JAK-STAT signaling pathway.

Key words : cell culture, endometrium, JAK, LIF, STATs, stromal cells

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INTRODUCTION

Leukemia inhibitory factor (LIF) is a pleiotropic cytokine. It appears from several lines of evidence that LIF plays important role in the process of blastocyst implantation in the primate (1, 2). In the human endometrium, expression of LIF reaches maximal levels during the secretory phase and is dependent upon progesterone dominance, highest amount being secreted by the glandular compartment (3–7). Laird et al (8) observed a gradual increase of LIF in uterine luminal fluid during days 7 to 12 after LH peak in proven fertile women, that was not found in women with unexplained infertility. Blockade of progesterone receptor action by high affinity progesterone receptor antagonist like mifepristone inhibits endometrial maturation and receptivity for blastocyst implantation along with repressed mid-luteal phase endometrial expression for LIF (3, 9). Furthermore, a functional role of LIF in blastocyst implantation has been proposed based on the evidence that failure of implantation occurs after administration of polyclonal antibody against LIF (anti-LIF Pab) or monoclonal antibody against LIF (anti-LIF Mab) in the uterus of monkeys during the peri-implantation stage (10, 11). Endometrial tissues from infertile women produced less LIF *in vitro* compared with that from fertile women (12). Discrete mutation in LIF gene has been shown to be associated with infertility in women (13). Collectively, it appears that implantation stage endometrial LIF may influence blastocyst implantation through autocrine-paracrine interaction (14). Although it has been indicated that LIF may act on different cell types via Janus kinase (JAK) and signal transducer and activation of transcription (STAT) signaling system (15–17), it is however not clear how LIF may influence the cellular physiology of mid-secretory phase endometrium in the primate. We have now examined the action of LIF in terms of cell proliferation, protein synthesis and secretion, and JAK signaling system in endometrial stromal cells obtained from long-term ovariectomized, hormone-simulated rhesus monkeys in the mid-secretory phase, and maintained in a three-dimensional primary culture on rat-tail collagen type I. We have earlier demonstrated that a three-dimensional primary culture system as employed in the present study provides a physiological platform for investigating cellular behaviour of mammalian endometrial cells (18–21).

MATERIALS AND METHODS

Animals and samples

Adult, healthy female rhesus monkeys (*Macaca mulatta*) were housed in the Primate Research Facility of the All India Institute of Medical Sciences. Eight monkeys were bilaterally ovariectomized under aseptic conditions as has been previously described (22) and used in the present study after 90 days of recovery period. This study was performed with the approval of the Ethics Committee for the Biomedical Use of Non-Human Primates of the All India Institute of Medical Sciences. Long-term ovariectomized rhesus monkeys (n=8) were subjected to hormone treatment for simulation of their menstrual cycles according to a procedure described previously (22). In the third simulated cycle, endometrial tissue samples were obtained through laparotomy on cycle day 18 following

a standard procedure described elsewhere (22, 23). Each animal was used at least three times for tissue collection after giving three rest cycles between successive surgeries. Immediately after tissue collection endometrial tissue samples were washed in ice-cold phosphate buffered saline (PBS) at pH 7.6, carefully minced on ice and enzyme digested with collagenase for the isolation of cells and stromal fibroblasts were separated on a percoll gradient. The procedural details have been given elsewhere (20, 21, 24). The estimated mean of cell viability in dye exclusion method was more than 90%, and that of cell purity was 97% based on immunocytochemical localization of vimentin in isolated cells.

Cell culture

Isolated monkey endometrial stromal cell-rich preparation ($4–5 \times 10^{5}.cm^{-2}$) were plated onto preformed rat-tail collegen type I gel matrix in 96-well flat bottom culture wells and cultured in DMEM-F12 (1:1) medium containing 5% fetal calf serum at 37°C in a humidified 5% carbon dioxide incubation chamber. The details of preparation of rat-tail type I collagen gel matrix and primary culture on collagen matrix have been given elsewhere (18–21, 24). After 48 h, cells were washed and cultured in serum-free, DMEM-F12 medium supplemented with insulin (1 g/l), transferrin (0.5 g/l), selenium (0.67 mg/l) and hydroxycorticosterone (50 µg/ml) for a further period of 24 h and used for the following experiments. The chemicals for cell culture were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA), GIBCO (Grand Island, New York, USA) and Invitrogen (Grand Island, New York, USA).

Protein synthesis by isolated endometrial stromal cells *in vitro*

The cultured endometrial stromal cells were subjected to treatment with rhLIF (R&D Systems, Minneapolis, USA) at concentrations of 0, 0.01, 0.1, 1.0, 10.0, 100, and 1000 ng/ml for 18 h in methionine-free DMEM-F12 medium containing ^{35}S-methionine (10 mCi/ml, BRIT, Hyderabad, India) and media supplements as described above. Cellular protein synthesis and secretion was assessed in cell lysates and in spent media from ^{35}S-methionine incorporated proteins precipitated by trichloroacetic acid following a method given elsewhere (24).

Cell proliferation *in vitro*

The cultured endometrial stromal cells were subjected to treatment with rhLIF at concentrations of 0, 0.01, 0.1, 1 and 100 ng/ml for 18 h in DMEM-F12 medium containing media supplements as described above. The effect of different doses of rhLIF on monkey endometrial stromal cell proliferation was studied from incorporation of BrdU by cells grown *in vitro* using the Cell Proliferation ELISA-BrdU Kit (Roche, Mannheim, Germany) as per the protocol provided by the manufacturer (25).

Intracellular signaling

Endometrial stromal cells grown *in vitro* were exposed for 15 min to rhLIF at different concentrations as shown in the Results section. Each experiment was repeated five times each in triplicate. Cell lysates containing 20 µg of protein from each group were subjected to 12% SDS-PAGE followed

by Western immunoblots (26, 27) for JAK1, STAT1, STAT2, STAT3 and pSTATs1-3 using their respective polyclonal anti-human antibodies obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, California, USA) and United Biotechnology, Inc. (Lake Placid, New York, USA). The bands were visualized using Vectastain ABC immunoperoxidase kit (Vector Laboratories, Burlingame, California, USA) and diaminobenzidine tetrahydrochloride (Sigma). Semi-quantitative analysis of the bands were done using gel scanner (GS670, Bio-Rad laboratories, Hercules, California, USA).

To study the specific cellular localization of JAK1 in endometrial cells exposed to different doses of rhLIF (0, 1, 100 ng/ml) endometrial stromal cells were cultured on collagen coated Lab-Tek chamber using the cell culture protocol as has been described above. Cells were exposed to rhLIF for 15 min and then fixed in cold paraformaldehyde (4%, w/v) for 15 min, permeabilized with Triton X-100, and immunostained using anti-JAK1 antibody (1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA) for 1 h at 37°C. Visualization was performed using Texas-Red conjugated with goat–antirabbit IgG, nuclei counterstained with DAPI (Invitrogen, Grand Island, New York, USA), and cells were then examined under confocal laser scanning microscope (Leica TCS SP2 with inverted Leica DMRE 2 microscope) (28).

Autophosphorylation of JAK1

Autophosphorylation of JAK1 in endometrial stromal cells was studied by exposing cells grown *in vitro* to different doses of rhLIF (0, 1, 10 or 100 ng/ml) for 15 min or to 1 ng/ml rhLIF alone for varying time periods (0, 5, 10 and 15 min) as described by Kodama et al (29). At the end of the experiment, cells were lysed in lysis buffer containing Tris-HCl (20 mmol/l, pH 7.4), NaCl (100 mmol/l), EDTA (5 mmol/l), Triton X-100 (1%, v/v), glycerol (10%, v/v), SDS (0.1%, w/v), deoxycholic acid (1%, w/v), NaF (50 mmol/l), phenylmethylsulphonylflouride (1 mmol/l), leupeptin (10 mg/ml) and aprotinin (10 mg/ml), and JAK1 was immunoprecipitated using anti-JAK1 antibody. The immunoprecipitate was isolated using protein G (Roche, Mannheim, Germany). The autophosphorylation activity of JAK1 was assayed by incubating immunoprecipitates with γ-^{32}P-ATP (10 µCi; BRIT, Hyderabad, India) in trizma base (25 mmol/l), NaCl (140 mmol/l), $MnCl_2$ (5 mmol/l), glycerol (10%, v/v) and ATP (20 mmol/l) for 20 min. After adding sample buffer and heating it at 95°C for 5 min, the samples were subjected to 12% SDS-PAGE and the gels were exposed to phosphor screen and scanned using phosphor imager (Bio-Rad Laboratories, Hercules, CA, USA).

Immunoneutralized LIF on endometrial stromal cell functions *in vitro*

Neutralization of rhLIF was done by pre-incubating rhLIF (1 ng/ml) with rhLIF-Mab (1 mg/ml) at 37°C for 60 min. In order to study the action of immunoneutralized rhLIF (inLIF) on endometrial stromal cell functions, cells were exposed either to rhLIF (1 ng/ml) or to inLIF complex for 15 min, and control group of cells were not exposed to either rhLIF or inLIF. The expression levels of JAK1, autophosphorylation of JAK1 and cell proliferation index in endometrial cells cultured *in vitro* were examined using the methodologies as described above.

Statistics

Comparisons between groups for different parameters were performed using analysis of variance (ANOVA) followed by multiple range test (30) using SPSS software. Values are shown as means ± SD.

RESULTS

Protein synthesis

Effect of different doses of rhLIF exposure on protein synthesis and secretion by monkey endometrial stromal cells is shown in Figure 1A. Increasing concentration of rhLIF *in vitro* resulted in a significant (P<0.01) decrease in cellular protein synthesis in isolated cells but with no marked change among groups treated with different concentration of rhLIF. There was no discernible change in the levels of secretory proteins in stromal cells grown in media with or without added rhLIF.

Cell proliferation

As shown in Figure 1B, with increasing concentration of rhLIF *in vitro* a progressive decrease (P<0.05) in cell proliferation index was obtained based on pooled data analysis. Significant (P<0.01) decrease was observed in cell proliferation indices in cells exposed to 1 and 100 ng/ml rhLIF compared with control (no rhLIF) and 0.01 ng/ml rhLIF treated cells.

JAK-STAT signaling

The expression of JAK1 was not detectable in isolated cells maintained *in vitro* in the absence of rhLIF, but its expression was found to increase in response to rhLIF added to cells *in vitro* as revealed from Western blot and confocal microscopic examination (Fig. 2). Autophosphorylation of JAK1 in endometrial stromal cells in culture showed no change with increasing concentration of rhLIF (Figs. 3A, B), however, cells showed increased (P<0.05) autophosphorylation with time of their exposure to rhLIF (Figs. 3C, D). STAT1,

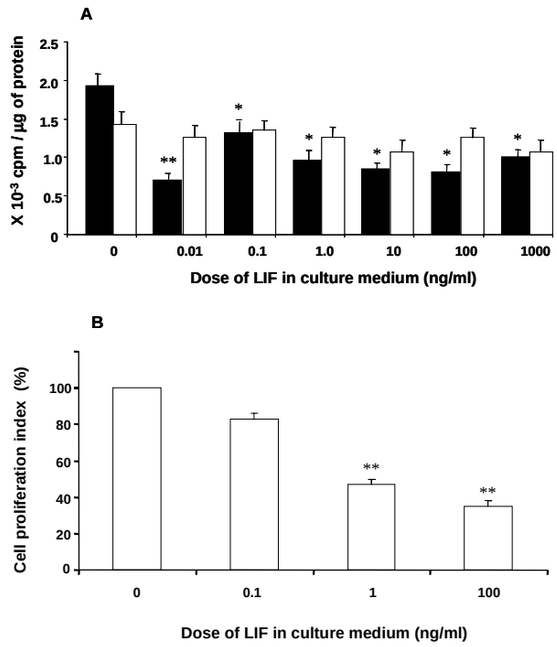

Fig. 1: Effect of different doses of rhLIF on *de novo* protein synthesis and secretion expressed in terms of ^{35}S-methionine incorporation in precipitable protein fraction (A) and cell proliferation index expressed as per cent BrdU positive cells (B). Endometrial cells showed decreasing trend in the cellular protein synthesis (*black bar*) with no change in the secretory protein (*white bar*) among different groups (A). Cell proliferation index assessed by BrdU incorporation showed a progressive decrease with increasing concentration of LIF (B). Values are shown as means ± SD. **P<0.01, *P<0.05 compared with control.

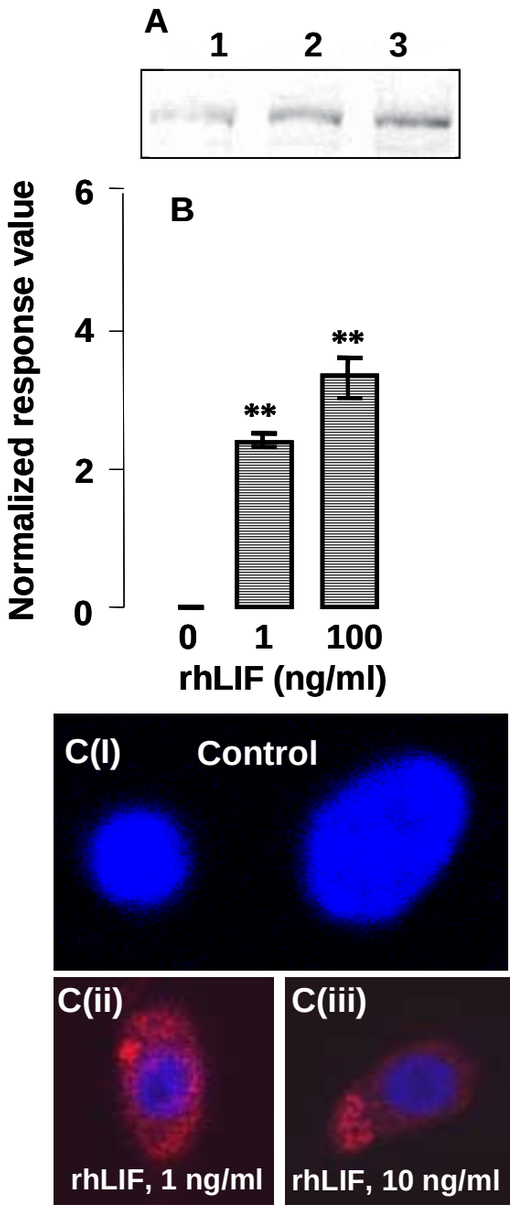

Fig. 2 : JAK1 expression as detected in Western immunoblot (A, B) and confocal laser scanning microscopy (C) in endometrial cells exposed to rhLIF at the concentrations of 0 [lane 1 in A and C(i)], 1 ng/ml [lane 2 in A and C(ii)], and 10 ng/ml [lane 3 in A and C(iii)] *in vitro*. Fig. C shows nuclei stained with DAPI (*blue*) and immunopositive JAK1 (*red*) in isolated endometrial stromal cells in three-dimensional primary culture.

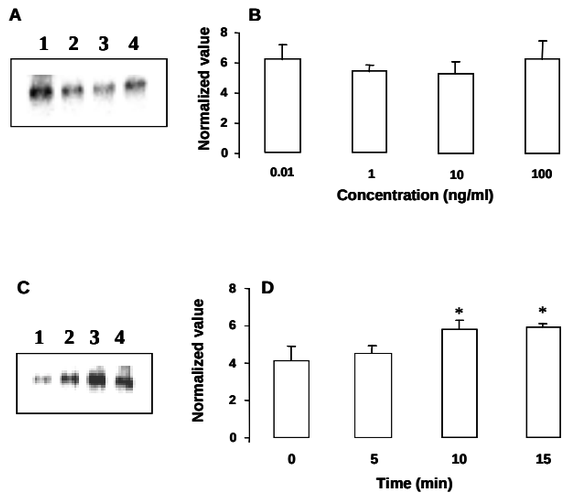

Fig. 3 : Effects of different doses (A, B) and time periods of exposure (C, D) to rhLIF on autophosphorylation of JAK1 in endometrial cells grown *in vitro*. Response values are shown in normalized scale based on integrated optical density and shown as means ± SD. *P<0.05 compared with values at 0 and 5 min.

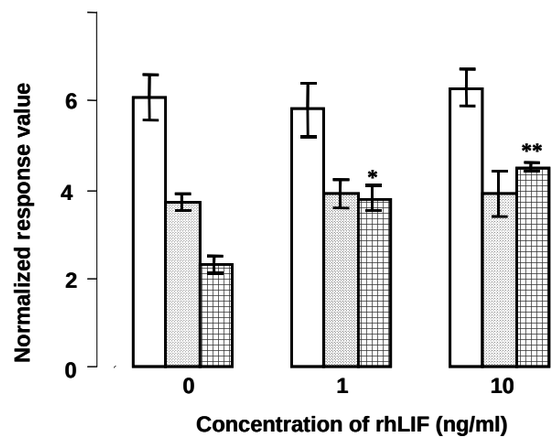

Fig. 4 : Pooled data from Western immunoblot analysis for STAT1 (*white bar*), STAT2 (*specked bar*), and pSTAT3 (*stripped bar*) in isolated endometrial stromal cells following exposure to different doses of rhLIF. Data are shown in normalized scale based on integrated optical density and as means ± SD. **P<0.01, *P<0.05 compared with control (0 ng/ml rhLIF) treatment.

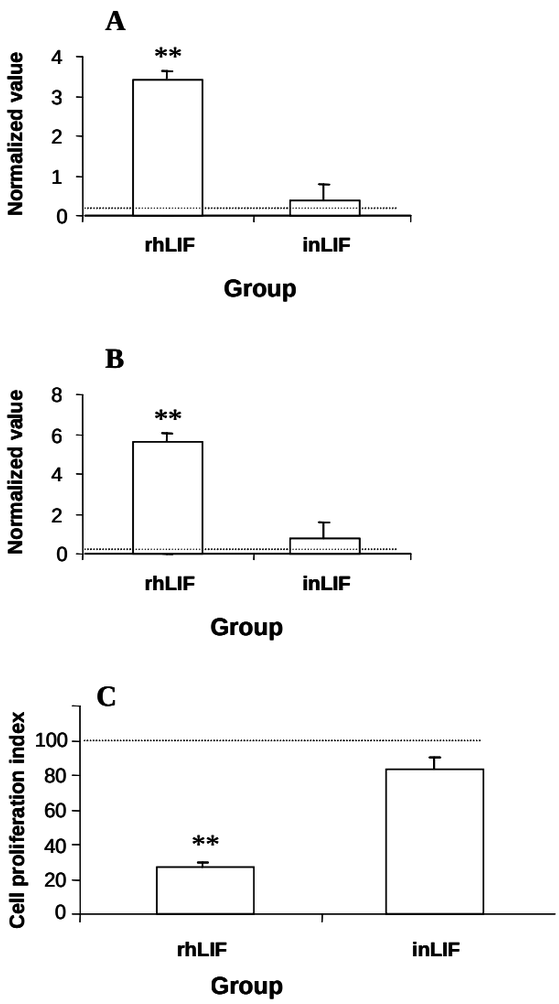

Fig. 5: Effects of rhLIF (1 ng/ml) and immunoneutralized rhLIF (inLIF) on expression (A) and autphosphorylation level (B) of JAK1 and proliferation index as seen by BrdU (C) in isolated endometrial stromal cells in three-dimensional primary culture. Values are shown as means ± SD. The dotted lines show control (no rhLIF) values.

STAT2 and pSTAT3 in isolated cells *in vitro* were detected by Western immunoblot, however, pSTAT1, pSTAT2 and STAT3 could not be detected. There was no change in expression of STAT1 and STAT2 with different doses of rhLIF, while pSTAT3 expression increased ($P<0.01$) with increased concentration of rhLIF in cell culture medium (Fig. 4).

Effect of immunoneutralized rhLIF (inLIF) on expression and autophosphorylation of JAK1 and cell proliferation index

The expression, and level of autophosphorylation of JAK1 significantly ($P<0.01$) decreased in cells following treatment with inLIF or no rhLIF compared with cells treated with rhLIF (Figs. 5A, B). However, cell proliferation index as examined by BrdU incorporation showed an increase ($P<0.01$) in cells exposed to either control (no rhLIF) or inLIF treatment as compared to cells exposed to rhLIF alone (Fig. 5C).

DISCUSSION

There are several reports indicating that leukemia inhibitory factor (LIF) is an important regulator of endometrial function, especially associated with blastocyst implantation (1–9, 31–33). Intrauterine administration of antibody to leukemia inhibitory factor (LIF) in monkeys during peri-implantation period resulted in inhibition of pregnancy establishment (10, 11). It has been suggested that luminal LIF derived from implantation stage blastocyst and endometrial epithelium acts on endometrial cells under progesterone dominance allowing endometrium to become receptive (14). Several reports have indicated the significant involvement of LIF at the endometrial epithelial level (10, 34–36). To our knowledge, there is no report indicating the action of LIF on the biological characteristics of implantation stage

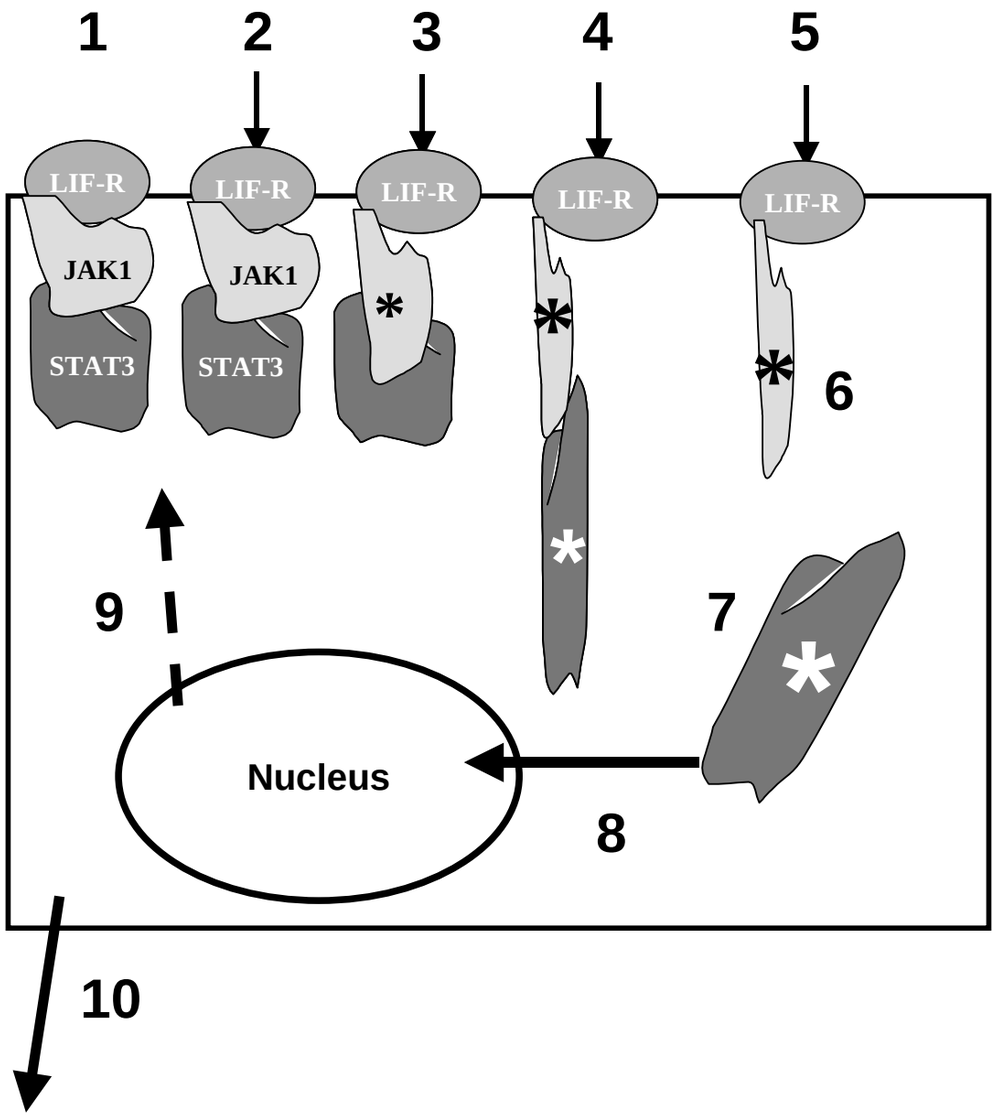

Fig. 6 : Proposed model of action of leukemia inhibitory factor (LIF) on mid secretory stage endometrial stromal cells in three-dimensional culture. In serum-free three-dimensional culture system, progesterone primed stromal cells express heterodimeric receptor molecules for LIF linked with cryptic JAK1/STAT3 signaling complex (step 1). Following binding with LIF, receptor molecules are activated (step 2) allowing activation of JAK1 molecules shown as a small black asterisk (step 3). The activation of JAK1 allows for increasing autophosphorylation along with change in naïve configuration. Such 'open' activated JAK1 molecules expose higher number of immunopositive domains shown as a large black asterisk (steps 4, 5). Activated JAK1 allows for phosphorylation of STAT3 molecules shown as white asterisks (steps 4–6), and dimerised pSTAT3 becomes free (step 7) that translocates to the nuclear compartment (step 8). Subsequent transcriptional activities may, on one hand, repress signaling networks at JAK-STAT level involving SOCS and other inhibitory peptides (step 9), and on the other hand, express specific sets of cellular behaviour (step 10). Our data suggest that LIF may help to suppress cell cycle and support cell survival processes in mid secretory stage stromal cells.

endometrial stromal cells in the primate.

In the present study, isolated stromal cell rich (97%) fraction obtained from mid secretory stage monkey endometrium was maintained on rat-tail collagen matrix and effects of recombinant human LIF (rhLIF) and immunonetralized rhLIF on cellular *de novo* protein synthesis, cell proliferation and the JAK-STATs were examined. We observed that administration of LIF reduced cellular protein synthesis and cell proliferation along with higher level of immunopositive JAK1 expression, its phosphorylation and immunopositive pSTAT3 compared with control (no rhLIF) treatment. However, no marked dose dependent property could be detected in these parameters except for the level of immunopositive JAK1. Interestingly, immunoneutralized LIF did not show any change in the expression level of immunopositive JAK1 and its phopsphorylation, as well as, proliferation index as compared to control treatment values. Although similar report is not available for implantation stage endometrial stromal cells, previous studies have indicated the putative involvement of LIF-LIFRβ-JAK1-STAT3 networks in diverse types of mammalian cells (15–17, 37–39), and that such networks may be operative in the process of growth inhibition in a few cell types (37, 40, 41). Figure 6 shows a tentative model of LIF action in stromal cells of receptive stage endometrium in the primate.

Collectively, it appears that rhLIF affects the JAK-STAT signaling pathway resulting in change in physiological behaviour of mid-secretory stage endometrial stromal cells grown in three-dimensional culture on rat-tail collagen matrix and that immunoneutralization of LIF led to its inhibition. While the overall observation from the present study does provide putative clues how intrauterine administration of antibody to LIF might have resulted in inhibition of pregnancy establishment (10, 11), there are a few relevant and interesting proximate and ultimate questions that also emerge.

In studies using a variety of cell types it is evident that LIF can increase the autophosphorylation level of JAK1 (16, 27, 42–45). We have also demonstrated similar increase in autophosphorylation of JAK1 in a timed manner following exposure of mid-secretory stage stromal cells to LIF and this action was blocked by immunoneutralization of LIF. However, it is not clear how the immunopositive level of total JAK1 increased from non-detectable level to fairly detectable level within 15 min after administration of LIF to stromal cells maintained under serum-free culture condition. Interestingly, immunofluorescent confocal laser scanning microscopy in the present study revealed (Fig. 2C) that prompt ‘capped’ orientation of immunopositive JAK1 molecules took place in cells treated with higher dose (10 ng/ml) of rhLIF compared with lower dose (1 ng/ml) of rhLIF treatment though the total optical densities did not show any marked shift. It is being suggested that JAK1 remains in a cryptic configuration linked with the receptor molecule complex for LIF in the absence of upstream activating agent in target cells under serum-free culture condition (46). Administration of LIF results in the activation of JAK1 associated with induction of ‘open’ configuration and cascade of autophosphorylation of the kinase molecule (Fig. 6). Further studies are

currently being done to test this hypothesis.

We have also observed that LIF induced JAK1 signaling in endometrial stromal cells was associated with phosphorylation of STAT3. It is however noteworthy that we could not detect unphosphorylated STAT3, while STAT1 and STAT2 could be detected in the target cells in primary culture irrespective of whether such cells were treated with LIF. Further studies may help to clarify whether such differences actually represent a biological behaviour or it was caused from any technical insufficiency.

In the present study, we have observed changes in proliferation index and protein synthesis, but with no marked dose dependent action in cells exposed to LIF. It is tempting to allude that such refractoriness of the target cells might be linked with pSTAT3 mediated activation of suppressor of cytokine signaling (SOCS) proteins (45, 47–49). Further experiments are to be performed using specific STAT3 inhibitor and SOCS inhibitors to address this question.

Although we did not investigate the teleonomy of observed cell behaviour with specific reference to endometrial receptivity, Nakajima et al (50) suggested that LIF supports the cell survival signals for endometrial stromal cells and may thereby protect the cells from trauma associated with blastocyst implantation.

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