0021-972X/96/$03.00/0
Journal of Clinical Endocrinology and Metabolism

Vol. 81, No. 2
Printed in U.S.A.

The Effect of Leukemia Inhibitory Factor (LIF) on Trophoblast Differentiation: A Potential Role in Human Implantation

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ABSTRACT

Leukemia inhibitory factor (LIF) is a multifunctional glycoprotein strongly associated with normal implantation in the mouse. We have recently determined that LIF is expressed in the human endometrium in a menstrual cycle dependent manner. Maximal expression is observed between days 19 and 25 of the menstrual cycle, coinciding with the time of human implantation. In this study we have utilized purified cultures of human cytotrophoblasts to examine the effects of LIF on several morphologic and biochemical markers of the trophoblastic differentiation. We purified human cytotrophoblasts from term placentae and cultured them with and without LIF (10 ng/mL). The secretion of human CG, oncofetal fibronectin, and progesterone were measured at 24, 48, 72, and 96 h. Northern blot analysis was used to assess messenger RNA (mRNA) expression of βhCG and oncofetal fibronectin. We found that LIF markedly decreased trophoblast production of hCG protein at 72 and 96 h, as well as expression of βhCG mRNA. LIF also significantly increased the expression of oncofetal fibronectin mRNA and secretion of the protein. LIF did not affect steroidogenic activity of cultured trophoblasts, as determined by progesterone production. These biochemical changes are characteristic of cytotrophoblast differentiation toward an anchoring extravillous phenotype. Thus, LIF appears to be an important regulator of human embryonic implantation by directly modulating trophoblast differentiation. (*J Clin Endocrinol Metab* **81:** 801–806, 1996)

LEUKEMIA inhibitory factor (LIF) is a glycoprotein that has multiple effects on different organ systems. It regulates the growth and differentiation of embryonic stem cells (1, 2), primordial germ cells (3), renal cells (4) peripheral neurons (5), osteoblasts (6), hepatocytes (7), and adipocytes (8). Recent studies have shown LIF to be strongly associated with normal implantation in the mouse. In the murine uterus, Bhatt *et al.* (9) found that LIF was maximally expressed 4 days after fertilization, the day of implantation. Elegant studies by Stewart *et al.* (10) point to the functional significance of LIF during murine implantation. They found that transgenic mice lacking LIF could produce normal embryos, but the embryos failed to implant. When embryos from LIF-deficient mice were transferred to the uteri of wild-type mice, normal implantation occurred. The implantation blockade was partially corrected with the infusion of LIF into the peritoneal cavity of the LIF-deficient mice, suggesting that endometrially produced LIF is critical for murine implantation.

Very little is known about the role of LIF in the human implantation process. We and others have recently determined that LIF is expressed in the human endometrium in a menstrual cycle dependent manner (11–13). We found maximal expression between days 19 and 25 of the menstrual cycle, coinciding with the time of human implantation. This temporal expression of LIF suggests a potential role for this cytokine in human implantation. One possible mechanism for this role could involve the regulation of trophoblast function and differentiation. In this study we have utilized purified cultures of human cytotrophoblasts to examine the effects of LIF on hCG secretion- a marker of villous syncytiotrophoblast differentiation-and on oncofetal fibronectin secretion-a marker of anchoring junctional trophoblast differentiation.

Received June 22, 1995. Revised August 22, 1995. Accepted August 29, 1995.

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Presented in part at the 41st Annual Meeting of the Society for Gynecological Investigation, Chicago, Illinois, March 22–25, 1994.

Materials and Methods

Cytotrophoblast preparation and culture

Human cytotrophoblasts were purified from the placentas of uncomplicated term pregnancies immediately after delivery by serial trypsin - DNase digestion followed by Percoll gradient centrifugation as described by Kliman *et al.* (14). Yields of viable trophoblast cells ranged from 60–150 × 10^6 cells/30 gram starting placental tissue. The purified cytotrophoblasts were cultured in Dulbecco's Modified Eagle's Media (DMEM) (Cell Biology media facility, Yale University) ± 10 ng/mL LIF (Becton Dickinson, Bedford, MA) with 2% fetal calf serum, 25 mmol/L glucose, and 25 mmol/L Hepes, supplemented with 4 mmol/L glutamine and 50 mg/mL penicillin, streptomycin, and neomycin (Gibco BRL, Grand Island, NY). The trophoblasts were plated in six well plates with 1 × 10^6 cells/well. One-half million trophoblasts were plated on 22 mm^2 cover slips in 35 mm wells for histologic examination, and 20 × 10^6 cells/dish were used for RNA extraction. Media were collected and changed every 24 h from each well or dish. After the collected media were spun at 2000 × *g* for 5 min, the supernatants were stored at -80 C for later hormonal analysis. One plate at each time point was frozen for DNA analysis. Each experiment was done in triplicate and was repeated at least three times.

Progesterone, hCG, LIF, and fibronectin immunoassays

Immunoreactive progesterone in the trophoblast culture media was quantified by RIA (Progesterone MAIA, Serono-Baker Diagnostics, Allentown, PA). According to the manufacturer, there is less than 1% cross-reactivity to other steroid hormones, the sensitivity for progesterone is 0.022 ng/ml and the intraassay, and interassay coefficients of variation are 8.06% and 7.71% respectively.

Immunoreactive hCG in the trophoblast culture media was quantified using the Serono hCG MAIACLONE immunoradiometric assay (CIBA-Corning Diagnostics, East Walpole, MA), with the results reported as mIU/mL (1st International Research Program). According to the manufacturer, the sensitivity of the test is reported as <1.0 mIU/mL, there is no measurable cross-reactivity to other hormones, and the intraassay and interassay coefficients of variation are 2.1% and 3.1%, respectively.

Immunoreactive LIF in trophoblast culture media was quantified using an enzyme linked immunosorbent assay from R&D Systems (Minneapolis, MN). According to the manufacturer, there is no measurable cross-reactivity with other known cytokines in this assay, the sensitivity for LIF is 2 pg/mL, and the intraassay and interassay coefficients of variation are 3.6% and 5.4%, respectively.

Immunoreactive oncofetal fibronectin secreted by trophoblasts was measured with quantitative enzyme-linked immunoassays and immunoblots as previously described by Feinberg *et al.* (15). Since the degree of trophoblast attachment has a marked effect on the quantity of oncofetal fibronectin secreted (16), actual basal levels of secreted fibronectin varied between different trophoblast preparations. Therefore, for each experiment, the unstimulated control media were normalized to 1.0, with LIF-stimulated samples measured and compared to the controls as a ratio. A similar analysis was previously carried out when determining the transforming growth factor-β dose-response stimulation of trophoblast oncofetal fibronectin (15).

Preparation of total RNA and Northern analysis

Total RNA was prepared by the guanidinium isothiocyanate-cesium chloride ultracentrifugation method of Chirgwin *et al.* (17). Total RNA (5 or 10 mg per lane) was size-fractionated by electrophoresis on 1% formaldehyde-agarose gels, transferred electrophoretically to Hybond-N^+ membrane (Amersham; Arlington Heights, IL), and cross-linked to the membrane by use of UV light. Prehybridization was performed for 5 h at 42 C in buffer comprised of 5x SSC, 5x Denhardt solution, formamide (50%, v/v), dextran sulfate (5%, w/v), NaH_2PO_4 (50 mM), and salmon sperm DNA (0.5 mg/mL). Hybridizations were performed for 16 h at 42 C in buffer composed of 5x SSC, 2x Denhardt solution, formamide (50%, v/v), dextran sulfate (10%, w/v), NaH_2PO_4 (20 mM), and salmon sperm DNA (0.1 mg/mL) with cDNA probes (5–15 mCi) complementary to βhCG and fibronectin mRNAs radiolabeled with [α-^{32}P]dCTP by random hexamer priming. The human βhCG cDNA (pCGβ474) used in this study was kindly provided to us by Dr. Irving Boime (Department of Pharmacology, Washington University, St. Louis, MO) and is complementary to full length βhCG (18). The human fibronectin cDNA (pGEM1Fnγ771) used in this study was kindly provided to us by Dr. Mon-Li Chu (Department of Biochemistry, University of New Jersey-Rutgers Medical School, Piscataway, NJ) and is complementary to the coding region (19). After hybridizing, the blots were washed with 1 x SSC and SDS (0.1%, w/v) for 15 min at room temperature, once with 0.1 x 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 (Rochester, NY). 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, Inc., Palo Alto, CA) radiolabeled with [α-^{32}P]dCTP by random hexamer priming. The autoradiographic bands were quantified by using a laser densitometer (Molecular Dynamics Inc., Sunnyvale, CA). Each β-hCG and fibronectin 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. Experiments were conducted on at least three different occasions with cells prepared from three different placentas and for each result a figure of an experiment, representative of all three experiments, is presented.

DNA analysis

The DNA content of each well was measured by DABA fluorescence using the method previously described by Kissane and Robbins (20).

Statistical analyses

Data are presented as the mean ± [sca]sd. Statistical analysis was performed with Student *t* test for pairs or with ANOVA with *post hoc* analysis (Tukey) for multiple comparisons.

Results

Effect of LIF on trophoblast DNA content and progesterone production

Upon plating, trophoblasts were maintained in culture medium with or without LIF (10 ng/mL). To analyze the effect of LIF on trophoblast cell number, DNA content was measured daily. DNA analysis showed that there was no difference in the DNA content per well in the LIF treated compared with the control groups at 24, 48, 72, and 96 h. Similarly, we found that LIF (10 ng/mL) treatment for 48, 72, and 96 h did not affect the level of the progesterone secretion by trophoblasts (Data not shown).

To investigate if trophoblasts produce LIF, we measured immunoreactive LIF in trophoblast culture medium. We did not detect LIF in supernatants of unstimulated or stimulated (by TGF-β [1 ng/mL] or IL-1α [10 U/ml]) trophoblasts (Data not shown).

Effect of LIF on hCG production by trophoblasts

The levels of hCG measured in the supernatants from cultured trophoblasts increased from 24 h to 72 h and plateaued at 96 h in both the LIF (10 ng/mL) treated and control groups. Very low levels of hCG were observed at 24 h in both groups (LIF: 0.68 ± 0.02 [mean ± SD] mIU/mg DNA; control: 0.82 ± 0.11 mIU/mg DNA). However, the levels of hCG in the LIF treated group were significantly lower than the control group at 48, 72, and 96 h. ($P < 0.01$ at 48 h; $P < 0.005$ at 72 and 96 h) (Fig. 1). The difference was marked at 72 h: 35.3 ± 8.5 mIU/mg DNA in the LIF treated group compared with 125.07 ± 7.2 mIU/mg DNA in the control group, a 3.5-fold difference. At 96 h, the level of hCG was 37.43 ± 8.3 mIU/mg DNA in the LIF treated group compared with 178.45 ± 18.4 mIU/mg DNA in the control group, a 4.8-fold difference. The inhibitory effect of LIF on the production of hCG was concentration-dependent between 0.01 and 1 ng/mL, but plateaued at higher concentrations (Fig. 2). The effects of 10 and 100 ng/mL LIF on hCG secretion was not significantly different than the 1 ng/mL concentration.

We also investigated whether this downregulation of hCG had occurred at the βhCG mRNA level. By Northern blot analysis we found that LIF treatment inhibited the expression of βhCG mRNA in trophoblasts in culture. The ratio of βhCG/G3PDH mRNA was consistently lower in cells treated with LIF, with an average 50% decrease seen in four experiments (Fig. 3).

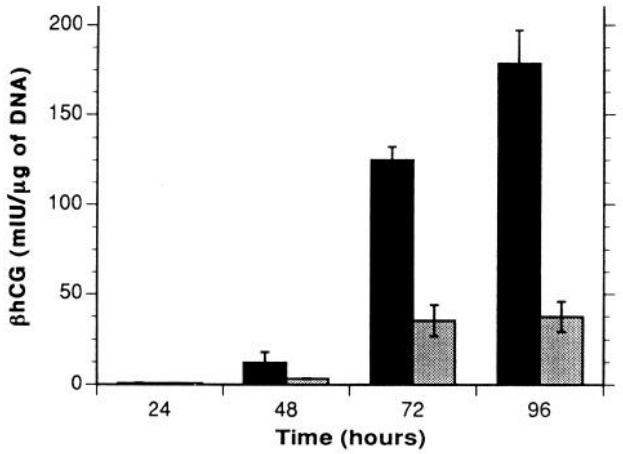

FIG. 1. Modulation of βhCG production by LIF in human trophoblast cells in culture. Trophoblasts were maintained in culture medium without (*black bars*) or with (*shaded bars*) LIF (10 ng/mL). The culture media were collected at 24, 48, 72, and 96 h, and βhCG was quantified by RIA. Data are from one representative experiment and are mean value of triplicate measurements for each group ± SD ($P < 0.01$ at 48 h; $P < 0.005$ at 72 and 92 h).

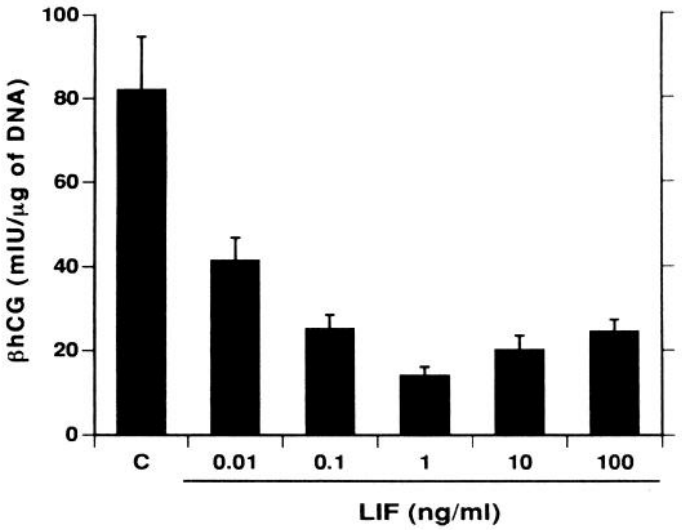

FIG. 2. Concentration-dependent modulation of βhCG production by LIF in human trophoblast cells in culture. Trophoblasts were maintained in culture medium without or with increasing concentrations of LIF (0.01 to 100 ng/mL). The culture media were collected at 72 h, and βhCG was quantified by RIA. Data are from one representative experiment and are mean values of triplicate measurements for each group ± SD ($P < 0.005$ between control and all LIF-treated groups).

Effect of LIF on oncofetal fibronectin production by trophoblasts

Treatment of trophoblasts in culture with LIF (10 ng/mL) led to increases in the levels of oncofetal fibronectin in a time-dependent manner. Although there was an increase in the oncofetal fibronectin secretion at 48 and 72 h, this increase was not statistically significant. At 96 h, there was an approximately 2-fold increase in the amount of oncofetal fibronectin secreted by the LIF treated trophoblasts ($P < 0.007$) (Fig. 4). LIF also increased the level of oncofetal fibronectin mRNA in cultured trophoblasts 2-fold (Fig. 5).

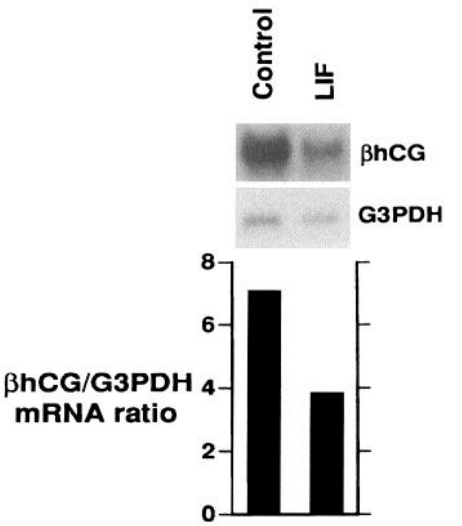

FIG. 3. Inhibition of βhCG mRNA by LIF in human trophoblast cells in culture. Trophoblasts were maintained for 72 h in culture medium without or with LIF (10 ng/mL), the medium changed every 24 h. At the end of the incubation period, total RNA was prepared from the cells. βhCG mRNA was evaluated by Northern analysis of total RNA (10 mg per lane) and expressed as a ratio of βhCG to G3PDH mRNA for each lane.

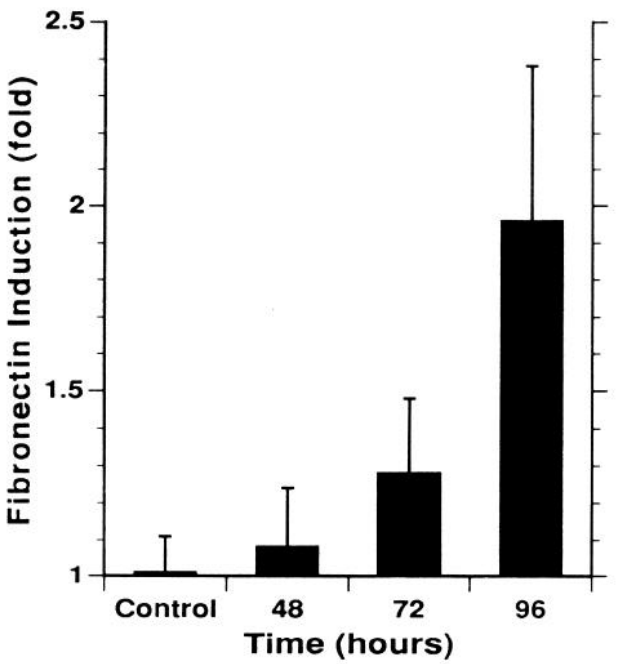

FIG. 4. Stimulation of human trophoblast fibronectin secretion. Trophoblasts were maintained in culture medium without or with LIF (10 ng/mL). The culture media were collected at 48, 72, and 96 h, and fibronectin was quantified by ELISA. Bars represent the ratio of fibronectin levels in the supernatants of LIF treated to control cells at each time point. Data are mean values of three experiments with triplicate measurements for each group ± SD ($P < 0.007$ at 96 h).

Effect of LIF on the trophoblast morphology

Because LIF appeared to be shifting the differentiation of the trophoblasts away from an hCG secreting phenotype and towards a fibronectin secreting phenotype, we investigated whether LIF treated trophoblasts would exhibit improved

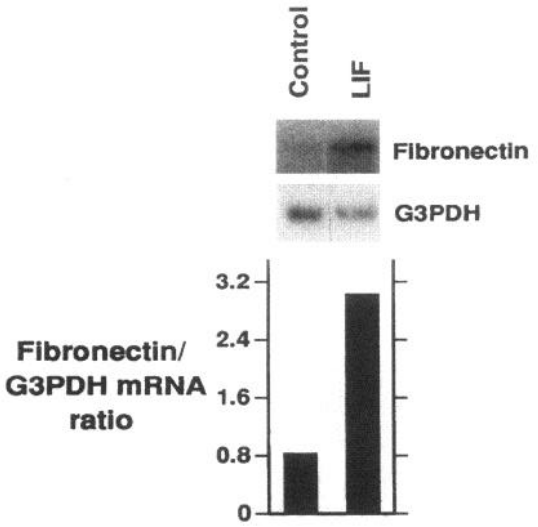

FIG. 5. Stimulation of human trophoblast fibronectin mRNA by LIF. Trophoblasts were maintained for 72 h in culture medium without or with LIF (10 ng/mL), medium changed every 24 h. At the end of the incubation period, total RNA was prepared from the cells. Fibronectin mRNA was evaluated by Northern analysis of total RNA (10 mg per lane) and expressed as a ratio of fibronectin to G3PDH for each lane. While fibronectin mRNA was stimulated by LIF, G3PDH mRNA remained unchanged or was decreased, as shown in this particular blot.

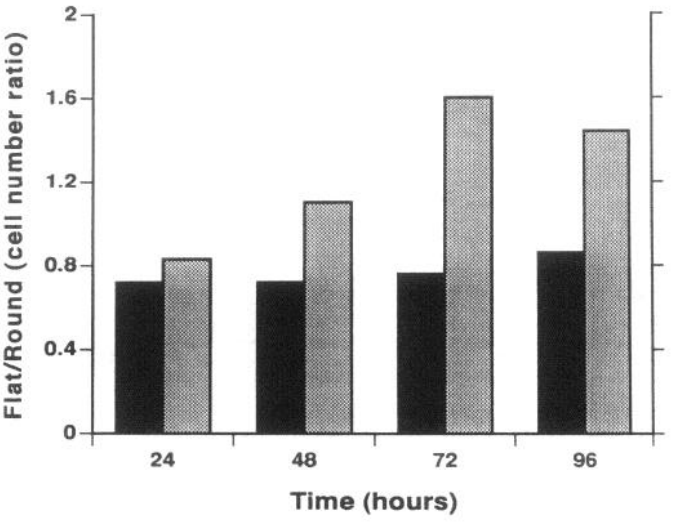

FIG. 6. Morphologic changes induced by LIF in human trophoblast cells in culture. Trophoblasts were maintained in culture medium without (*black bars*) or with (*shaded bars*) LIF (10 ng/mL). Trophoblasts were morphologically assessed at 24, 48, 72, and 96 h, and the ratio of round to flat cells was quantified. Data are from one representative experiment and are mean values of triplicate measurements for each group. ($P < 0.01$ at 48 h; $P < 0.005$ at 72 and 92 h).

spreading on the culture surface as a result of an increased production of extracellular matrix (ECM) around the cells. Because increased cell-ECM interaction results in increased cell flattening, we evaluated this hypothesis by assessing the ratio of flat to round trophoblasts at 24 h intervals with or without added LIF. The ratio of flat to round cells increased starting from 48 h in the LIF (10 ng/mL) treated group compared with control group ($P < 0.01$ at 48 h; $P < 0.005$ at 72 and 92 h) (Fig. 6).

Discussion

Successful implantation depends upon a complex interaction between the developing blastocyst and the endometrium. It is known that for human implantation to occur, trophoblasts must attach to the underlying endometrial surface epithelium. The trophoblasts then interdigitate between the endometrial cells, travel through the basement membrane, and ultimately invade the maternal spiral arteries (21). The factors that control the intricate cascade of molecular and cellular events are beginning to be elucidated; however, they remain incompletely understood.

There is growing evidence that successful implantation depends upon regulation of growth factors and cytokines acting in an autocrine/paracrine fashion. These factors, in conjunction with steroid hormones, constitute maternal-embryonic communication. Our developing understanding of human implantation has shown many similarities to the mouse. In this model, LIF has been shown to be one of the essential cytokines for implantation (10). A role for LIF in human implantation is suggested because we and others have found LIF in the human endometrium and have seen increased amounts of LIF in the secretory phase of the menstrual cycle (11–13). Relevant to these observations, LIF receptor mRNA expression has been found in human blastocysts (12). Therefore it seems likely that LIF is important for early uterine-blastocyst communication in the human, as shown in the mouse. While mouse and human implantation differ in some significant ways (most notably in that human implantation involves invasive trophoblasts and mouse does not), a common feature between mouse and human implantation is the necessity for tight attachment of the placenta to the uterine lining. While in the mouse LIF may function as the major cytokine that promotes the attachment of the blastocyst and the developing placenta to the uterus, in the human LIF may be one of several factors that serve this same function.

One mechanism of LIF action could be through control of trophoblast differentiation. Depending on the external environment, undifferentiated cytotrophoblasts can differentiate along three pathways to become: 1) villous syncytiotrophoblasts, 2) extravillous anchoring trophoblasts, or 3) invasive intermediate trophoblasts (22) (Fig. 7). There is growing evidence that growth factors and polypeptides may mediate these differentiation pathways (23). Biochemical and cellular markers of the trophoblast differentiation pathway have been established. The villous syncytiotrophoblast produces hCG as well as other essential pregnancy hormones (24). *In vitro* experiments have shown that cAMP (25–27), EGF (28), and hCG (29) direct cytotrophoblast differentiation towards a hormonally active syncytiotrophoblast phenotype and upregulate hCG production. The second type of differentiated trophoblast is the junctional trophoblast found where the chorionic villi make contact with the extracellular matrix (ECM). These cells form the anchoring cell columns seen at the junction of the placenta and the endometrium. Recently, a specific type of oncofetal fibronectin, trophouteronectin (TUN), has been identified at the tropho-

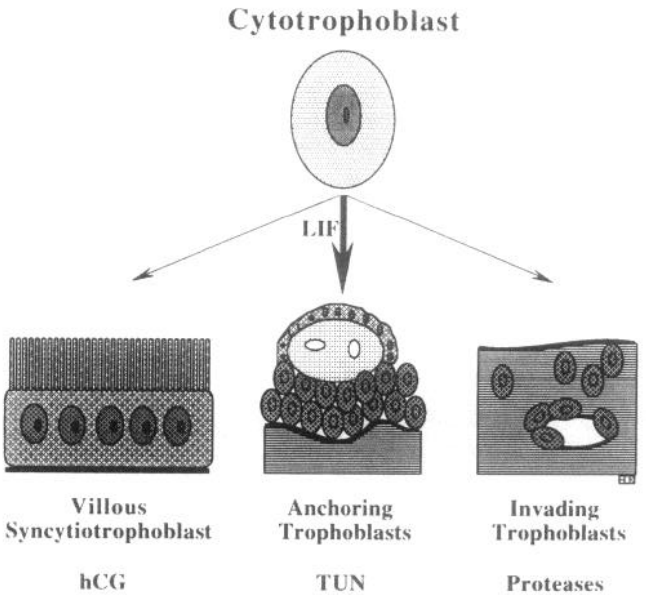

FIG. 7. Pathways of trophoblast differentiation. Just as the undifferentiated basal layer of the skin gives rise to differentiated keratinocytes, the cytotrophoblast (the stem cell of the placenta) gives rise to the differentiated forms of trophoblasts. (*Left*) Within the chorionic villi, cytotrophoblasts fuse to form the overlying syncytiotrophoblast. The villous syncytiotrophoblast makes the majority of the placental hormones, the most studied being hCG. Cyclic AMP and its analogs, and more recently hCG itself (29), have been shown to direct cytotrophoblast differentiation towards a hormonally active syncytiotrophoblast phenotype. (*Center*) At the point where chorionic villi make contact with external extracellular matrix (decidual stromal ECM in the case of intrauterine pregnancies), a population of trophoblasts proliferates from the cytotrophoblast layer to form the second type of trophoblast-the junctional trophoblast. These cells form the anchoring cell columns that can be seen at the junction of the placenta and endometrium throughout gestation. Similar trophoblasts can be seen at the junction of the chorion layer of the external membranes and the decidua. The junctional trophoblasts make a unique fibronectin (trophouteronectin [TUN]) that appears to mediate the attachment of the placenta to the uterus. TGF-β, and now from our current work, leukemia inhibitory factor (LIF), has been shown to downregulate hCG synthesis and upregulate TUN secretion. (*Right*) Finally, a third type of trophoblast differentiates towards an invasive phenotype and leaves the placenta entirely-the invasive intermediate trophoblast. In addition to making human placental lactogen, these cells also make gelatinases, urokinase-type plasminogen activator (u-PA) and type 1 plasminogen activator inhibitor (PAI-1). LIF, in addition to upregulating TUN production and downregulating hCG secretion, has recently been shown to also downregulate trophoblast gelatinolytic activity (44).

blast-ECM junction and is thought to be a glycoprotein responsible for attachment of anchoring trophoblasts to the endometrium (30). TGF-β has been shown to increase TUN production while decreasing hCG production (15, 31), stimulating trophoblast differentiation toward the anchoring phenotype. The third pathway leads to the development of invasive trophoblasts, which interdigitate through the extracellular spaces of the endometrium and penetrate the maternal spiral arteries. These trophoblasts produce a variety of proteases (32–34) and protease inhibitor (35), which may mediate the invasive process. Unlike hCG, progesterone is produced and secreted by cytotrophoblasts as well as syncytiotrophoblasts (36), and is produced as early as 24 h by trophoblasts in cell culture (26). Thus progesterone secretion appears to be independent of the trophoblast differentiation pathway, and, consistent with this, we did not observe an effect of LIF on the progesterone production by cultured trophoblasts. We also found LIF did not affect the DNA content of trophoblasts, suggesting that LIF is not cytotoxic or mitogenic in this system.

Our results suggest that LIF may affect which differentiation pathway trophoblasts are directed towards. Exposure of cultured trophoblasts to LIF resulted in a decrease in βhCG mRNA and protein with a concomitant increase in fibronectin mRNA and TUN secretion. This pattern of response to LIF suggests that this cytokine, like TGF-β (31), shifts the trophoblast differentiation pathway away from the hormonally active syncytiotrophoblast phenotype towards the anchoring junctional trophoblast phenotype. Recently, Sawai *et al.* (37) reported that LIF stimulates hCG production by trophoblasts. These investigators examined the effect of a short LIF exposure (150 min), while we examined the long-term effects of LIF (24–96 h). There may be a biphasic response to LIF, initially a stimulatory effect and thereafter an inhibitory effect as a result of cytotrophoblast differentiation; a similar phenomenon is observed for the effects of cAMP analogs on urokinase-type plasminogen activator (34). Although we have mostly used 10 ng/mL concentration of LIF to demonstrate its effect on trophoblast differentiation, we have also shown that this effect is observed at 0.01 ng/mL concentration, a physiologic dose secreted by stimulated endometrial stromal cells (11).

The pivotal role of LIFs ability to shift trophoblast differentiation towards a TUN-secreting anchoring phenotype is supported by our recent report of the expression of TUN by 4–8 cell human embryos (38) and the expression of an endometrial integrin (39) that may bind to this trophoblast fibronectin at the time of trophoblast penetration of the endometrium. The model that emerges from this work suggests that, as the early conceptus begins to implant into the endometrium, embryo-derived cytokines and growth factors, such as IL-1 (40), TNF-α (41), PDGF (42), and TGF-β (43), further stimulate endometrial stromal cells to produce LIF (11), which in turn may induce the implanting trophoblasts to secrete increased amounts of TUN. This trophoblast-endometrial positive feedback system may help to drive the implantation cascade, ensuring progressive attachment of the blastocyst to the endometrium.

Acknowledgment

The authors wish to thank Erika L. Meaddough and Cai-Liang Wang for their skillful technical assistance.

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