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 Chorionic Gonadotropin (hCG), 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 hCG and oncofetal fibronectin. We found that LIF markedly decreased trophoblast production of hCG protein at 72 and 96 h, as well as expression of phCG 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 trophoblast differentiation and on 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).
Progestrone, hCG, LIF, and fibronectin immunoassays

Immunoreactive progestrone in the trophoblast culture media was quantified by RIA (Progestrone MAIA, Serono-Baker Diagnostics, Allentown, PA). According to the manufacturer, there is less than 1% cross-reactivity to other steroid hormones, the sensitivity for progestrone 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 MAIA CLONIC immunoradiometric assay (CIBA-Corning Diagnostics, East Walpole, MA), with the results reported as mIU/mL (1st International Research Program). According to the manufacturer, there is no measurable cross-reactivity to other hormones, and the sensitivity for hCG is reported as <1.0 mIU/mL, with 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 immunosorbent assays 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), NaH2PO4 (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), NaH2PO4 (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-32P]dCTP by random hexamer priming. The human βhCG cDNA (pCG874) 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 (pGENM1F171) 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 human fibronectin cDNA. After hybridization, 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-32P]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 ± [sc]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 whether 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 4.9-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).
Effect of LIF on oncotel fibronectin production by trophoblasts

Treatment of trophoblasts in culture with LIF (10 ng/mL) led to increases in the levels of oncotel fibronectin in a time-dependent manner. Although there was an increase in the oncotel 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 oncotel fibronectin secreted by the LIF treated trophoblasts (P < 0.007) (Fig. 4). LIF also increased the level of oncotel fibronectin mRNA in cultured trophoblasts 2-fold (Fig. 5).
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.

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-
Cytotrophoblast

LIF

Villous Syncytiotrophoblast

Anchoring Trophoblasts

Invading Trophoblasts

hCG

TUN

Proteases

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 column 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 (trophoentericin [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).

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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 cytrophoblast 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.

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References

5. Yamamori T, Fukuda K, Aebersold R, Korschning S, Fann MJ, Patterson PH. 1989 The cholinergic neuronal differentiation factor from heart cells is identical...


