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LEUKEMIA INHIBITORY FACTOR (LIF) ENHANCES TROPHOBLAST DIFFERENTIATION MEDIATED BY HUMAN CHORIONIC GONADOTROPIN (hCG)

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Summary: We investigated the effect of LIF on the differentiation of trophoblasts. Isolated cytotrophoblasts were cultured with and without LIF and cell smears were immunocytochemically analyzed, using anti-hCG antibody. The percentage of differentiated trophoblasts stimulated by 10ng/ml of LIF was about 2.5-fold that in the control culture. The effect of LIF in inducing the differentiation of cytotrophoblasts to syncytiotrophoblasts was dose-dependent. The same effect was shown when hCG was added to the medium. This enhancing effect of LIF on trophoblast differentiation was blocked by adding anti-hCG antibody to the culture system. These results indicate that LIF enhanced trophoblast differentiation by stimulating hCG production in trophoblasts, and not by exerting a direct effect on the trophoblasts.

Human placental villi consist of an outer layer of trophoblasts and an inner layer of connective tissue elements, including macrophages, fibroblasts and fetal blood vessels. The trophoblast layers consist of an inner layer of mononuclear cytotrophoblasts and an outer layer of multinuclear syncytiotrophoblasts. It is generally accepted that the cytotrophoblast is the mitotically active, undifferentiated stem cell and that the syncytiotrophoblast is the mitotically inactive, differentiated end-stage cell derived from the cytotrophoblast. It is thought that the growth of chorionic villi is dependent on the morphological differentiation of cytotrophoblasts to syncytiotrophoblasts. The differentiation of trophoblasts is thought to be followed by fusion of their plasma membranes. However, apart from this finding, the details of this differentiation remains unknown.

It has been revealed that several cytokines, such as epidermal growth factor (EGF) (1) and transforming growth factor-β1 (TGF-β1) (2), are involved in the differentiation of trophoblasts in an autocrine and/or paracrine fashion. As well as these

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cytokines, hCG has also been reported to play a role in trophoblast differentiation (3), a role considered to be mediated by hCG/LH receptors (4). The production of hCG is reported to be regulated by trophoblast-derived cytokines such as IL-6 (5), IL-1 (6), tumor necrosis factor-α(TNF-α) (7), and transforming growth factor-β_1(TGF-β_1) (8). We have recently shown that leukemia inhibitory factor(LIF), which shares a signal transducing molecule with IL-6, also enhances the production of hCG (9). This finding indicates that LIF may also be related to the differentiation of trophoblasts. LIF is a 40 to 60-kD glycoprotein that has been shown to be essential for blastocyst implantation in a mouse reproduction system (10). However, it is not clear how LIF plays a role in implantation and in the maintenance of pregnancy. In this study, we attempted to precisely analyze the morphological differentiation of cytotrophoblasts to syncytiotrophoblasts by immunocytochemistry, using anti-hCG antibody, and to clarify the way in which LIF influences trophoblast differentiation.

Materials and Methods

Reagents. Highly purified human chorionic gonadotropin (hCG) was purchased from Sigma (St. Louis, MO). The anti-hCG antibody(Ab) used was a monoclonal mouse antibody to human chorionic gonadotropin (ZYMED, Carlton Court, San Francisco). Recombinant(r) LIF was purchased from Genzyme (Cytokine Research Products, Cambridge, MA).

Preparation of cytotrophoblasts. Fresh placentas were obtained from legal abortions at 7-9 weeks of gestation after the informed consent of the patients was given. Single-cell suspensions of cytotrophoblasts, prepared by enzyme treatment of placental tissues with type III collagenase (Funakoshi Co., Tokyo, Japan) and DNase (Sigma, St. Louis, MO) at 37 °C for 30 min, applied to a 60% Percoll (Pharmacia, Piscataway, NJ) sedimentation gradient as described previously (9). Cells at the interface were then washed twice with culture medium to remove collagenase. Cell viability, assessed by the trypan blue dye exclusion test, was found to exceed 90%. The cells ($1x10^5$/well) were cultured in RPMI 1640 with 10% FCS(Summit, Greeley, Colo) at 37 °C in 12-well flat-bottomed microplates(Falcon, Becton Dickinson, Bedford, MA)

Stimulation of trophoblasts by hCG or LIF. Cytotrophoblasts were stimulated in medium containing 50mIU/ml hCG or various concentrations of rLIF in the presence or absence of 10mg/ml anti-hCG Ab. Each experiment was performed with three different placentas to obtain reproducible results.

Immunocytochemical analysis. For immunocytochemical analysis at the single-cell level to determine the localization of hCG and its appearance in nuclei and plasma, trophoblast cells were detached from culture plates and cell suspensions were thoroughly washed and applied to a Cytospinner (Shandon Southern Products; Cheshire, UK) (11). The cells on the slides were treated by the avidin-biotin-peroxidase complex (ABC) method (Omnitags, Lipshow, Pittsburgh, PA) as described previously (12). Ten mg/ml of anti-hCG monoclonal antibody was used as the first antibody.

Classification of trophoblasts and cell counting. The classification of trophoblasts was assessed by direct microscopic examination at a magnification of X 200. The cell counting began at a random point near the middle of the coverslide where differentiation was most obvious, and all immunostained cells, regardless of the level of immunostaining intensity, were counted. A total of 300 cells was counted at random in each experiment. Mononuclear cells that were not stained were classified as cytotrophoblasts. Multinuclear cells that were positively stained were classified as syncytiotrophoblasts. Mononuclear cells with positive staining were classified as transitional trophoblasts. All experiments were repeated two or three times on different placentas, with triplicate performances within each experiment. Subjectivity was minimized by this counting strategy.

Statistics. Results were assessed by analysis of variance, followed by Student's t-test or Duncan's new multiple range test for multiple comparison. $P< 0.05$ was considered to be significant.

Results

Trophoblast differentiation by immunocytochemical analysis. Microscopically, cytotrophoblasts isolated from first trimester placentas were small, round and not stained by anti-hCG Ab (Fig.1A). The cytoplasm and nucleus were a little enlarged and the nuclear/cytoplasmic ratio had increased during culture. Simultaneously, the cells became positively stained (Fig.1B). We classified these cells as transitional trophoblasts. These results indicated that these cells had differentiated into syncytiotrophoblasts that had multiplenuclei and expanding cytoplasm and showed strong staining (Fig.1C).

Effect of hCG and LIF on trophoblast differentiation. We investigated the effect of hCG and LIF on trophoblast differentiation, using the immunocytochemical analysis described above. In this analysis, we examined the differentiation of trophoblasts that changed from cytotrophoblasts to transitional trophoblasts and then to syncytiotrophoblasts (Fig. 1). In the control medium on day 2, the percentage of cytotrophoblasts was 77%, that of transitional trophoblasts was 18%, and that of syncytiotrophoblasts was 5% (Fig. 2). Figure 3A shows the cytospin smear of trophoblasts cultured in the control medium on day 2. As shown in Figure 2A, 50mIU/ml of hCG enhanced trophoblast differentiation, resulting in the percentage of transitional trophoblasts increasing to 42%($p< 0.0001$vs. control) and the percentage of syncytiotrophoblasts increasing to 13%($p< 0.0001$vs. control). When cytotrophoblasts were cultured with the same amount of hCG and 10mg/ml of anti-hCG Ab, hCG-induced trophoblasts differentiation was decreased to the control level. Figure 2B shows the dose-dependent effect of rLIF-induced trophoblast differentiation in medium containing various concentrations of rLIF. The effect of rLIF on cytotrophoblast differentiation to transitional trophoblasts became apparent at a concentration of 10ng/ml ($p< 0.0001$vs. control) and its effect on the differentiation to syncytiotrophoblasts became apparent at a concentration of 1.0ng/ml ($p< 0.05$vs. control). Figure 3B shows a cytospin smear of trophoblasts stimulated by 10ng/ml rLIF. The effect of 10ng/ml rLIF-induced trophoblast differentiation was completely blocked by an appropriate amount of anti-hCG Ab, as shown in Figure 2C. When trophoblasts were cultured in medium containing LIF and anti-hCG Ab, the percentage of transitional trophoblasts was 3%($p< 0.0001$vs. the LIF-stimulated group) and the percentage of syncytiotrophoblasts was 19%($p< 0.0001$vs. the LIF-stimulated group). The use of murine IgG instead of anti-hCG Ab did not influence the effect of LIF on trophoblast differentiation (data not shown). This result indicates that the trophoblast differentiation induced by LIF is dependent on the effect of hCG. Figure 3C shows a cytospin smear of trophoblasts stimulated by rLIF and anti-hCG Ab.

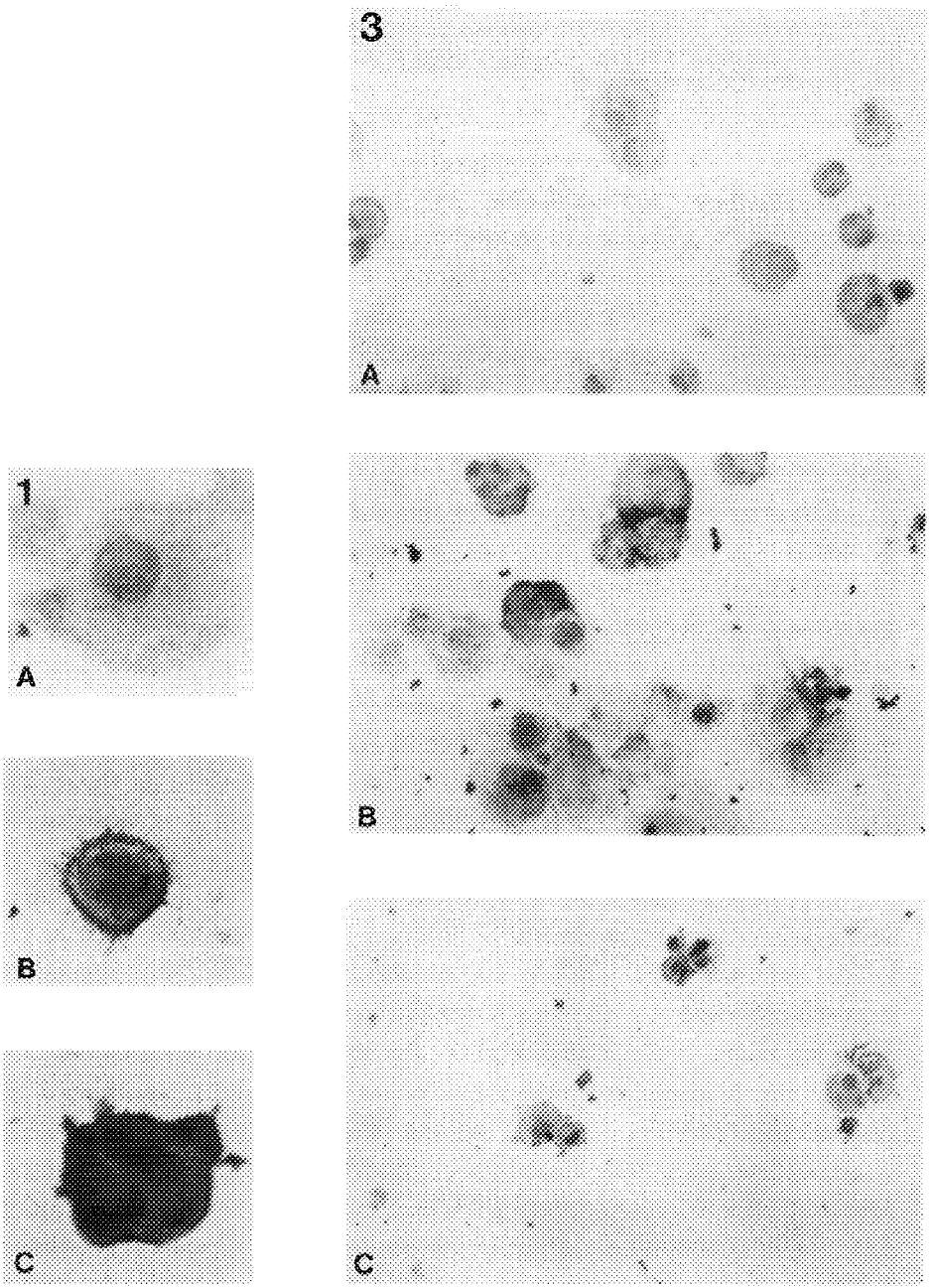

Figure 1. Immunocytochemical demonstration of three types of trophoblasts. (A) cytotrophoblast; this has a single nucleus and is not stained by anti-hCG Ab. (B) "transitional trophoblast", in which the cytoplasmic and nuclear volume have increased a little and the N/C ratio is increased, weak positive staining is shown. (C) syncytiotrophoblast; these are multinuclear, have expanding cytoplasm, and are strongly stained. Magnifications: (A) X400, (B) and (C) X200.

Figure 3. Cytospin smears. (A) Smear of trophoblasts cultured in the control medium. (B) Smear of trophoblasts stimulated by 10ng/ml of rLIF. Transitional trophoblasts and syncytiotrophoblasts were increased. (C) Smear of trophoblasts cultured with LIF and anti-hCG Ab. At this point, transitional trophoblasts and syncytiotrophoblasts had completely vanished.

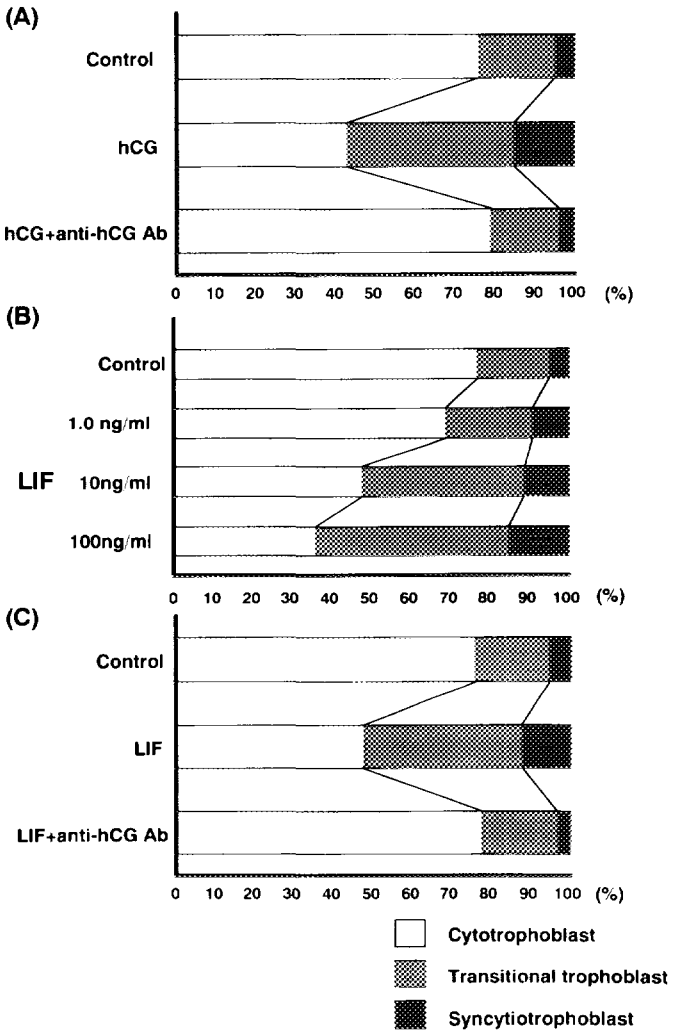

Figure 2. Percentages of cytotrophoblasts, transitional trophoblasts, and syncytiotrophoblasts. (A) Cytotrophoblasts were cultured with 50mIU/ml hCG or hCG and anti-hCG Ab. The percentages of the three different types of trophoblasts were assessed by immunocytochemical analysis. The increase in the percentage of hCG-stimulated differentiated trophoblasts was blocked in medium containing anti-hCG Ab. (B) Cytotrophoblasts were cultured in medium containing various concentrations of rLIF; dose-dependent effects of rLIF-induced trophoblast differentiation are shown. (C) The effect of rLIF on increasing the percentage of differentiated trophoblasts was completely blocked when trophoblasts were cultured with both rLIF and anti-hCG Ab.

Discussion

The main object of this study was to investigate the effects of hCG and LIF on the precise process of trophoblast differentiation from cytotrophoblast to syncytiotrophoblast. Yagel *et all.* (13) showed that trophoblast-derived hCG acted as a growth factor, since trophoblast proliferation was reduced in the presence of anti-hCG antibody. Shi *et al.* (3) showed that hCG regulated the differentiation of cytotrophoblasts into syncytiotrophoblasts, however they regarded differentiated

trophoblasts as cytotrophoblast aggregations without intervening plasma membranes. Contrary to their idea, we consider differentiated trophoblasts to be those that are positively stained by anti-hCG Ab, or alternatively trophoblast aggregations without intervening plasma membranes, but showing positive staining. Our immunocytochemical analysis showed the existence of transitional trophoblasts. These were derived from cytotrophoblasts and subsequently differentiated into syncytiotrophoblasts. These transitional trophoblasts **(I)** had a clear singlenucleus, **(II)** were positively stained by anti-hCG Ab, which is weaker than syncytiotrophoblasts, and **(III)** fused gradually, differentiating into syncytiotrophoblasts. The existence of "transitional trophoblasts" is in agreement with the result of the study of Hoshina *et al.* (14), who reported that transcription of the hCGα gene in trophoblasts was initiated before the completion of syncytial formation. The percentage of these differentiated trophoblasts (transitional trophoblasts and syncytiotrophoblasts) shown in this present study was greater than the percentage of fused trophoblasts, estimated by a method described in previous studies (3). Shi *et al.* (3) also found that a trophoblast differentiation response was seen at 50mIU/ml hCG. This reached maximal level at 500mIU/ml, and then declined. The increase in the percentage of differentiated trophoblasts, mediated by hCG, was blocked by anti-hCG Ab.We observed similar tendencies in our analysis system.

The production of hCG is reported to be stimulated by various cytokines (5-8). LIF has been shown to enhance hCG production in trophoblasts in a dose-dependent manner through glycoprotein(gp) 130, an IL-6 signal transducing molecule, and this hCG production was mediated by tyrosine kinase (9). Decidua produce much higher levels of LIF mRNA and protein than chorionic tissue (9,15). Stewart *et al.* considered LIF to be essential for blastocyst implantation, however, this function of LIF is not sufficiently elucidated. We attempted here to elucidate novel function of LIF in the differentiation of cytotrophoblasts to syncytiotrophoblasts. At the implantation site, trophoblasts invade the decidual basalis and then form chorionic villi, the growth of these chorionic villi being dependent primarily on the morphological differentiation of cytotrophoblasts to syncytiotrophoblasts. Another intriguing function of LIF was demonstrated by Barnard *et al.* (16),who revealed that LIF brought about a marked increase in the rate of myoblast proliferation, but that it did not enhance the fusion of myoblasts to form myotubes. However, in this study, we demonstrated that LIF enhanced trophoblast differentiation in a dose-dependent manner, which was equivalent for the fusion of cytotrophoblasts. This increase in trophoblast differentiation was completely blocked by anti-hCG, indicating that this effect of LIF was mediated by hCG. Therefore, we suggest that LIF produced by decidual cells may be involved, via the production of hCG, in stimulating the development of the trophoectoderm into trophoblasts, and the differentiation of cytotrophoblasts into syncytiotrophoblasts at the implantation site and in early pregnancy. In this study we elucidated the hCG-dependent function of LIF, and we are now investigating other functions of LIF that are independent of hCG.

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