Studies on the Effect of Thyroid Hormone and Epidermal Growth Factor on the Cultured Human Cytotrophoblast

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Abstract. We have previously reported that human placental cytotrophoblasts (C-cells) contain nuclear 3,5,3'-triiodo-L-thyronine (T_3) receptors. Using a C-cell culture system, the present study was undertaken to clarify some of the effects of T_3 and EGF on trophoblastic cells. C-cells were purified from human term placenta by treatment with trypsin-DNase and percoll gradient centrifugation aggregated, then fused, differentiating into multinuclear syncytiotrophoblasts (S-cells) with incubation times up to 96 h *in vitro*. As the incubation time increased, the number of immunocytochemically reactive cells with antibodies to hCG- α , hCG- β and hPL increased. Anti-EGF antibody reacted only with the initial C-cells, while anti-EGF receptor antibody reacted only with fused S-cells. Maximum secretion of hCG and hCG- α by the cultured cells was evident only when the cells were cultured in T_3 (10^{-8} M) or EGF (10 ng/ml) containing medium. When the initial cells were exposed to 10^{-8} M T_3 from 0 to 48 h of incubation, the secretion in 48–96 h was significantly accelerated. However, exposure from 48 to 96 h had no effect on peptide excretion. Although an exposure of these cells to 10 ng/ml EGF during 48–96 h of incubation stimulated the secretion of hCG and hCG- α , 0–48 h exposure did not produce any positive effect regardless of incubation time. These results indicated that the main target cell of T_3 is the C-cell, while that of EGF is the S-cell. Furthermore, it is suggested that the interaction between T_3 and its receptor facilitated functional cell differentiation. It is possible to propose a paracrine/autocrine control mechanism in which EGF synthesized by C-cells acts on S-cells.

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THE HUMAN villous trophoblast is composed of the cytotrophoblast (C-cell) and the syncytiotrophoblast (S-cell). C-cells, which have a proliferative character, fuse and differentiate into multinucleated S-cells [1–3]. The non-proliferative S-cell possesses essential functions such as production and secretion of steroid and protein hormones for the maintenance of pregnancy [4–6]. Although the control mechanism in the trophoblast has not yet been elucidated, there have been reports that differentiation and proliferation in some mammalian cells are induced by a mechanism

of receptor-mediated endocytosis of epidermal growth factor (EGF) [7], which is capable of stimulating hCG secretion by cultured human choriocarcinoma cells [8] and perfused human placenta [9]. The cell membrane of the human S-cell is rich in receptors for a number of growth factors including those for EGF [7], insulin [10], and the family of insulin-like growth factors (IGFs): IGF-I and IGF-II [11, 12], and somatomedin A and C [13, 14].

In previous studies, we demonstrated that human placental trophoblasts contain nuclear 3, 5, 3'-triiodo-L-thyronine (T_3) binding sites biochemically similar to those isolated from rat liver [15], and that these binding sites are mainly localized in the nuclei of C-cells [16]. Furthermore, in our culture system for trophoblastic cells the initially harvested

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C-cells changed morphologically and functionally into S-cells as the incubation time increased up to 120 h [16].

EGF stimulates hCG secretion in trophoblast [17, 18]. Human placenta and isolated trophoblast cells are rich in EGF receptor [19]. It is suggested that EGF should perform possible physiological roles in fetoplacental development. From the viewpoint of the mechanism of action of growth factors, the present study was undertaken to elucidate any differences in action between T_3 and EGF, focusing on human term placental trophoblasts which morphologically and functionally change as incubation time increases.

Materials and Methods

Chemicals and buffers

3, 5, 3'-Triiodo-L-thyronine (T_3), trypsin and fetal calf serum (FCS) came from Sigma Chemical Co. (U.S.A.). Human EGF (homogeneous grade) was purchased from Gibco Lab., Inc. (U.S.A.). These and the other chemicals used were of analytical grade.

Isolation and culture of cytotrophoblast

Human cytotrophoblast was isolated from human term placenta, as described previously [16] by a method which was originated by Kliman *et al.* [2] with slight modification. In short, freshly obtained placental trophoblasts digested with 0.125% trypsin and 0.4 mg/ml DNase I in Mg^{++} and Ca^{++} free Hunk's solution (Handai Biken, Osaka) at 37°C for 30 min was pelleted and further purified by Percoll gradient centrifugation. The mononuclear cells with a density of about 1.031 g/ml were harvested and were adjusted to a concentration of 5×10^5 cells/ml. This was done by adding DMEM-H-G (Dulbecco's MEM containing 50 mM glucose and 50 mM HEPES, Handai Biken) supplemented with 50 μ g/ml Tobramycin and 10% FCS. The harvested cells were then plated into Lab-Tek tissue culture 8 chambered-slides (Nunc, Inc., U.S.A.). The culture chambers were incubated with or without T_3 or EGF in a humidified 5% CO_2 -95% air atmosphere at 37°C for 96 h. Every 24 h of incubation, the culture medium was withdrawn by gentle aspiration and frozen at -40°C for later assays of human chorionic gonadotropin (hCG) and hCG- α subunit, and then

recharged with fresh medium. At designated times the chambers and gaskets were removed, and the slide was washed with 10 mM potassium phosphate buffer, pH 7.5, containing 0.15 M NaCl (buffer A), and fixed with Bouin's solution for 15 min. Following fixation, the slides were washed with buffer A at room temperature. Later, these were immersed in buffer A at 4°C for up to 4 days, for further cytological and immunocytochemical studies. Cell viability on day 5, as assessed by trypan blue dye exclusion, was 95%. hCG and hCG- α concentrations secreted into the media were determined by an enzyme immunoassay (Elmotec hCG, Mochida Pharmaceutical Co.), and by a radioimmunoassay [20], respectively. Cross-reactivity of the anti-hCG- α to hCG was 0.2%, and that of the anti-hCG to hCG- α was negligible. Therefore, hCG values interfering with the hCG- α RIA were ignored in the calculation of hCG- α .

Immunocytochemical staining of the cultured cells

The Biotin-Streptavidin A Amplified System Kit (Biogenex Lab., U.S.A.) was used according to the biotin-streptavidin-peroxidase complex method. Mouse anti-human EGF monoclonal antibody and mouse anti-EGF receptor monoclonal antibody (Transformation Research, Inc., U.S.A.) were used as the primary antibodies, and both diluted at 1:1,000. The Percoll-purified mononuclear cells and the cultured cells attached to the slides were stained with hematoxylin and eosin. The residual cells were prepared for immunocytochemical staining. Non-specific immunoglobulin binding sites were blocked by 30 min preincubation with 5% goat serum. Non-immune rabbit serum as the control was applied for 60 min. Biotin-linked mouse immunoglobulin was used for the second antibody.

Results

As shown in Fig. 1, in our culture system the initially harvested mononuclear cells began to fuse together and only 20% of the mononuclear cells which had adhered to the bottom of the culture chamber remained single at 24 h of incubation. Subsequently, the total number of mononuclear cells gradually decreased and about 3% remained unchanged at 96 h. Fused cells increased to 15% of the total number of cells at 24 h, 42% at 48 h, 70%

Fig. 1. Morphological changes in the cultured cells with the lapse of incubation time. Wide columns of single (■), aggregate-type (□) and fused cells (▨) represent population ratios in the total number of cells. Narrow columns denote ratios of immunoreactive cells for hCG- α (▧), hCG- β (▩) and hPL (▪) in respective wide columns.

Fig. 2. Effect of T₃ and EGF on hCG secretion by cultured cells. The harvested mononucleated cells were incubated for 96 h in the presence of various concentrations of T₃ or EGF. Every 24 h the medium was changed to a fresh one, and at 96 h of incubation the hCG concentration in the medium was assayed by an enzyme immunoassay as described in "Materials and Methods". Each bar represents the mean $\pm$ SD for 6 replicates. Data were compared by the paired *t* test. * *p* < 0.01 vs control. ** *p* < 0.05 vs control.

at 72 h and 90% at 96 h, similar to the results obtained by Kliman *et al.* [2]. Immunoreactive hCG- α was found in 15% of the initially harvested mononuclear cells, while neither hPL nor hCG- β was recognized in these cells. At 48 h and thereafter, the ratio of the cells, aggregate or

fused, which reacted immunocytochemically with anti-hCG- α and anti-hCG- β was relatively similar, reaching $\geq 90\%$ at 96 h of incubation. On the other hand, immunoreactive hPL was already recognized in 30% of the single and 90% of the aggregate-typed cells at 24 h of incubation.

Fig. 3. Time course effect of T₃ and EGF on cultured cells. (a) Addition of T₃ to the media for the first 48 h had nearly the same effect on hCG and hCG-α secretion as the entire 96-h incubation with T₃. (b) The addition of EGF (10 ng/ml) for the latter 48 h had nearly the same effect on hCG and hCG-α secretion as the entire 96-h incubation with EGF. Each bar represents the mean ± SD for 6 replicates. *p < 0.01 vs control.

As shown in Fig. 2, the rate of secretion of hCG into the media by the cultured cells between 72 and 96 h of incubation was dose-dependently stimulated by 10^{-10} – 10^{-8} M T₃. However, a higher concentration (10^{-7} M) T₃ suppressed hCG secretion as compared to the control level ($p < 0.01$). EGF (0.1–100 ng/ml) also stimulated hCG secretion (at least $p < 0.05$). The maximum stimulation was observed at 10^{-8} M T₃ or 10 ng/ml EGF ($p < 0.01$, respectively). Any concentration of T₃ or EGF used in the present study induced an increase in neither the total number of nuclei nor DNA content, and no further morphological changes in the cultured cells were seen (data not shown).

Next, the initially harvested C-cells were treated with 10^{-8} M T₃ or 10 ng/ml EGF for the first half

(0 to 48 h) or the latter half (48 to 96 h) of the incubation time. The addition of T₃ to media for the first 48 h had nearly the same effect on hCG and hCG-α secretion as that by treatment with T₃ for the whole 96 h. During the latter half of incubation, exposure to T₃, however, did not increase the secretion rates of hCG and hCG-α to the control level (Fig. 3-a). On the other hand, EGF at the optimal concentration added to the medium from the start and maintained throughout, or between 48 to 96 h incubation, increased both hCG and hCG-α secretion rates at and after 72 h of incubation. Secretion rates of these proteins were not stimulated when the cultured cells were exposed to EGF for only the first 48 h (Fig. 3-b).

The initially harvested C-cells were positively

Fig. 4. Immunocytochemical staining with anti-EGF and anti-EGF receptor antibody (original magnification: $\times 400$). The initially harvested C-cells (a and b) and the multinuclear fused cells cultured for 72 h (c and d) were immunocytochemically stained for EGF (a and c) and EGF receptor (b and d), as described in "Materials and Methods".

stained and the multinucleated S-cells cultured for 72 h were negatively stained with anti-EGF antibody. On the other hand, anti-EGF receptor antibody reacted positively with S-cells and negatively with C-cells (Fig. 4).

Discussion

As discussed in the previous paper [16] and also in the present study, initially harvested mononuclear cells morphologically resembled C-cells in light microscopy. These cells aggregated and transformed into multinuclear cells during up to 96 h of incubation. More than 80% of the fused cells, especially at and after 72 h of incubation, developed the ability to synthesize hCG- α , hCG- β and hPL. These results indicated that mononuc-

lear C-cells differentiated into multinuclear S-cells via aggregated intermediate-type cells. It is of interest that not only a greater part of the aggregated cells but also mononuclear cells (although the number of these decreased drastically following incubation) also reacted with antibodies to these peptides. It has been reported that cells of a morphology intermediate between that of C- and S-cells have been found [1, 3]. Fifteen percent of the initial mononuclear cells reacted only with anti-hCG- α , suggesting that, strictly speaking, these cells might belong to the intermediate-type. The current definition of cultured C-cells as distinguished from S-cells is vague. It is generally admitted that the initially harvested or cultured mononuclear single cell is a C-cell [2, 18, 21]. However, even though there were remaining unaggregated mononuclear cells which

reacted immunocytochemically with antibodies to hCG- α , hCG- β and hPL, these cells should not be regarded as C-cells, because the C-cell of the normal placenta is described as rich in free ribosome but possessing very little endoplasmic reticulum where these hormones are synthesized [22]. These cells should perhaps be more critically defined on the basis of their respective functions and also electron-microscopic findings.

In the culture system used in the present study, the addition of T_3 to the medium at the start of incubation facilitated hCG and hCG- α secretion after a lag period of 48 h. A similar effect was reproduced by T_3 added in the first 48 h of culture, while exposure to T_3 in the latter half failed to reproduce the same effect. On the other hand, EGF exhibited a similar stimulatory effect if added to the medium at and after 48 h of incubation. EGF added in the first 48 h could not induce these effects. Nulsen *et al.* [23] reported a similar stimulatory effect of EGF (10 ng/ml) on hCG secretion but it was after a much longer incubation time than 96 h.

The present study, which used 10 ng/ml EGF over 4 days of culture, could not confirm the conclusion of Morrish *et al.* [18] who observed morphological differentiation of C-cells to S-cells induced by 100 ng/ml EGF. They used mononuclear cells which had been precultured in FCS-free medium for 6 days or more. It is possible that this difference in findings may be explained by the fact that the mononucleated cells in the study of Morrish *et al.* [18] had already differentiated into intermediate trophoblastic cells or had acquired the functional character of S-cells by the time EGF was added to the medium on the 6th day of preculture. This possibility may be entertained because since day 1 of preculture the mononucleated cells had been secreting hPL, thereby exhibiting one characteristic of intermediate trophoblastic cells even before exposure to EGF. Moreover, if the increased secretion of hCG and hPL by the precultured cells was interpreted as induced by EGF, then such an interpretation assumes that the precultured cells have a sufficient amount of EGF receptors. However, in term placentas, the notable presence of EGF receptors is a characteristic of S-cells and not of C-cells [24]. Therefore, while EGF stimulated hCG and hPL secretion in the study of Morrish *et al.* [18], this effect might be unrelated to the ability or inability of EGF to

induce morphological changes in cultured mononucleated cells. DePalo and Das [21] have demonstrated that EGF stimulates EGF-receptor biosynthesis in cultured C-cells. This, however, would explain the ability of their precultured cells to react with EGF. They observed the EGF effect in C-cells cultured in 20% FCS medium, while Morrish *et al.* maintained an FCS-free medium. Furthermore, Morrish *et al.* [18] exposed the culture to 100 ng/ml ($\approx$ 17 nM) of EGF, a concentration which DePalo and Das [21] found stimulate the degeneration, not the production, of EGF receptors in cultured C-cells. In such an environment, it would be difficult to understand how, as reported by DePalo and Das, EGF could induce EGF receptor biosynthesis in cultured C-cells. This area, therefore, requires further examination.

At any rate, because the increased expression of hCG and hCG- α induced by T_3 and EGF was not accompanied by morphological changes, the present study suggests that the target cell of T_3 is the C-cell, while the S-cell is the target of EGF. Our findings also suggest that in term placenta, the interaction between T_3 and its receptor facilitates functional cell differentiation without C-cell proliferation. At term, the S-cells are terminally differentiated and no longer have the capacity to divide [25]. It should be further clarified whether thyroid hormone increases EGF receptor and EGF receptor mRNA levels in trophoblast, as mentioned concerning liver [26, 27]. EGF has also been shown to decrease nuclear T_3 binding in pituitary GH₄ cells in culture [28]. Further investigation of hormone modulation of T_3 , EGF and their receptors should be of great interest.

Although the present immunocytochemical results cannot be ascribed to a lack of EGF receptors on the initial C-cells, our data may support a part of a similar thesis, proposed by Yamamoto *et al.* [29]: a paracrine or autocrine control mechanism in which EGF synthesized by C-cells facilitates certain functions, including the induction of EGF-receptor action, of neighboring S-cells or S-cell differentiated from the C-cells themselves. It follows that further studies clarifying EGF synthesis in the C-cell should be undertaken.

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