The effects of growth factors on human normal placental cytotrophoblast cell proliferation

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The effects of growth factors were investigated on the proliferation of a normal placental cytotrophoblast cell line (NPC). Epidermal growth factor (EGF), transforming growth factor alpha (TGFα) and insulin-like growth factor-I (IGF-I) stimulated NPC cell proliferation. In contrast, TGFβ1 was found to be a negative regulator, inhibiting EGF-induced cell proliferation. When EGF/TGFα receptor was analysed by radio-ligand binding, two binding sites of different affinities were revealed in the proliferating NPC cells but only the low affinity binding site was detected in the non-proliferating cytotrophoblast cells in primary cultures. The results suggest that EGF stimulates cytotrophoblast proliferation through high affinity binding sites.
Key words: epidermal growth factor/epidermal growth factor receptor/insulin-like growth factor-I/placental cell proliferation/transforming growth factor-α

Introduction

The placenta is the intermediate organ between the mother and the fetus during pregnancy. It develops from the trophectoderm of the blastocyst and the endometrium of the mother. During the early implantation period, the trophectoderm invades the uterine endometrium and gives rise to cytotrophoblast cells (Boyd and Hamilton, 1970; Hamilton and Hamilton, 1977). The cytotrophoblast cells replicate rapidly during the first trimester and many of them differentiate into syncytiotrophoblast cells. These proliferating cells extend from the forefront of the growing chorionic villi as cytotrophoblast columns which become anchored to the uterus (Boyd and Hamilton, 1970; Hamilton and Hamilton, 1977). During the last 10 years, a variety of growth factors and their receptors have been found in the placenta (Jonas *et al.*, 1985; Liu *et al.*, 1985, Chen *et al.*, 1988; Frolik *et al.*, 1983; Goustin *et al.*, 1985) and in the decidua (Han *et al.*, 1987; Tamada *et al.*, 1991). Previously, we have found that addition of epidermal growth factor (EGF) and insulin stimulated human chorionic gonadotrophin (HCG) and progesterone secretion by cultured human cytotrophoblast cells (Li and Zhuang, 1991). More

recently, we have established a normal placental cytotrophoblast (NPC) cell line from the first trimester human placental tissue (Li *et al.*, 1996). This cell line was continuously maintained in serum-free medium supplemented with defined growth factors. NPC represents undifferentiated, proliferating cytotrophoblast cells. The mononuclear cells enable us to directly count in real terms the cumulative increase in cell numbers induced by growth factors. The present paper describes the regulation of NPC cell proliferation by EGF, transforming growth factor alpha (TGFα), insulin-like growth factor I (IGF-I) and TGFβ1.

Materials and methods

Materials

Mouse EGF of tissue culture grade or receptor grade was purchased from Collaborative Research Co, Bedford, USA. IGF-I, TGFα, and TGFβ1 of tissue culture grade were purchased from Gibco BRL, Grand Island, NY, USA.

Primary culture of cytotrophoblast cells

Human placental tissues of 6–8 weeks gestation were obtained from the maternity ward of Haidian Hospital, Beijing, China. The isolation and primary culture of the cytotrophoblast cells have been described elsewhere (Li *et al.*, 1991). To obtain the NPC cell line, the purified cells were seeded into collagen-coated 24-well multiplates (Corning, NY, USA) at 2×10^5 cells/well with 1 ml of Ham's F12/Dulbecco's modified Eagle's medium 1:1 (FD medium, Gibco BRL) containing 10 ng/ml EGF (Tissue culture grade, Collaborative Research Co), 10 μg/ml insulin (Sigma Chemical Co, St Louis, MO, USA). 0.1% bovine serum albumin (BSA; Sigma), 2 mM glutamine (Dongfong Chemical Co., Shanghai, China), 1.75 mM HEPES (Sigma), 100 IU/ml penicillin and 100 μg/ml streptomycin (regular medium) and cultured at 37°C in 5% CO_2:95% air. After 24 h of culture, the cells were washed twice with FD medium and fresh medium was added containing appropriate growth factors. For EGF binding analysis, the cells were then cultured without EGF for 24 h.

Cell proliferation test

NPC cells of passages 10–20 were used in this experiment. The cells were maintained in regular medium (Li *et al.*, 1996) with 10^{-6} M dexamethasone (Sigma). To determine the effect on cell numbers, NPC cells were seeded at 2×10^4 cells/well in 24-well multiplates in 0.5 ml FD medium containing 0.1% BSA and growth factors were added at different concentrations and in different combinations at four wells/group. The cells were placed in incubator for 48 h. The cells were then detached from the plastic by trypsinization and counted with a Coulter counter (Coulter Electronic Inc, Hialeah, FL, USA). All experiments were repeated at least three times, although only one set of results is presented. Data are shown as mean ± SE. Tests of significance were carried out using Student's *t*-test.
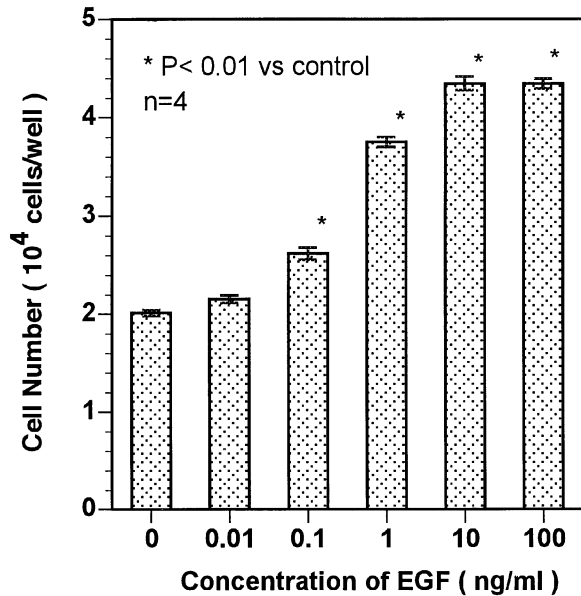
Figure 1. Stimulation of normal placental cytotrophoblast (NPC) cell proliferation by epidermal growth factor (EGF). NPC cells were seeded at 2×10^4 cells/well with 10 µg/ml insulin, 0.1% bovine serum albumin (BSA) and 10^{-6} M dexamethasone. Epidermal growth factor (EGF) was added at different concentrations to the cultures at plating. The cells were incubated for 48 h and then dissociated into single cells by trypsinization and counted with a Coulter counter. Data are shown as mean $\pm$ SE of four replicate cultures and were tested for significance using Student's *t*-test.

EGF binding

Mouse EGF (Collaborative Research Co.) was labelled with Na [^{125}I] (Du Pont, Boston, MA, USA) using Chloramine T (Sigma Chemical Co.) according to Hunter *et al.* (1962) and purified with Sephadex G-25 (Pharmacia LKB, Upsala, Sweden) filtration. The labelled EGF displayed specific radioactivity at ~170 µCi/µg. The NPC cells were grown in 24-well multiple well plates until the cell number had reached ~2×10^5 cells/well. They were washed twice with FD and cultured without EGF for a further 24 h. Then the cells were washed twice with warm FD medium before [^{125}I]-EGF (in FD medium containing 0.5% BSA) was added to triplicate wells at different concentrations with or without 1 µg EGF. Incubation was carried out at 37°C for 1 h. The cells were then washed with FD medium five times and lysed by incubation with 1 N NaOH at 50°C for 1 h. Finally the lysates were transferred into test tubes and the radioactivity in each tube was measured by a γ-counter (Beckman, Palo Alto, CA, USA).

Results

Effect of EGF on NPC cell proliferation

The NPC cells were established in serum free medium in the presence of EGF. When they were cultured in the absence of EGF, most cells remained quiescent and the cell number showed little increase for up to 5 days. The addition of EGF to the culture induced rapid cell replication. The increase in cell number in EGF-treated cultures became obvious after 24 h of treatment and became more pronounced after 48 h (Figure 1). EGF at the concentrations of 0.01–100 ng/ml increased cell number in a concentration-dependent manner. Maximal stimu-
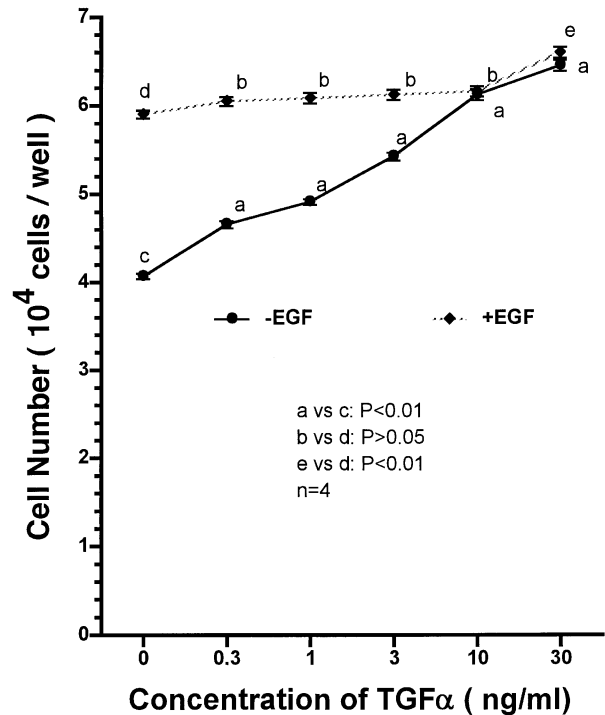

Figure 2. Effect of transforming growth factor α (TGFα) on normal placental cytotrophoblast (NPC) cell proliferation. NPC cells were seeded at 2×10^4 cells/well with 10 µg/ml insulin, 0.1% bovine serum albumin (BSA) and 10^{-6} M dexamethasone in the presence or absence of epidermal growth factor (EGF) (10 ng/ml). TGFα was added at different concentrations. The cultures were incubated for 48 h before trypsinization and cell counting. Data are shown as mean $\pm$ SE of four replicate cultures and were tested for significance using Student's *t*-test.

lation was observed at a concentration of 10 ng/ml when the cell number was doubled (Figure 1).

Effect of TGFα on NPC cell proliferation

TGFα was tested for its ability to replace EGF as a stimulator of NPC cell proliferation, since it was known to bind the EGF receptor. When cells were treated with TGFα at concentrations of 0.3–3.0 ng/ml in the absence of EGF, cell growth was enhanced in a concentration-dependent manner. The maximal stimulation was observed at a concentration of 30 ng/ml. The cell number reached 6.5×10^4 representing a 1.5-fold increase over the control. However, treatment with various concentrations of TGFα in the presence of 10 ng/ml EGF did not cause stimulation unless the TGFα concentration was much higher than that of EGF (Figure 2).

EGF binding sites

Specific EGF binding sites exist in both NPC cells and in cytotrophoblast cells in primary cultures. The binding of [^{125}I]-EGF to cells of both primary cytotrophoblast cultures and NPC cultures was saturable and in competition with binding by unlabelled EGF. Scatchard analysis showed a single class of binding site (Kd = 2.01×10^{-9}/M) in the primary cytotrophoblast cultures (Figure 3). In contrast, two classes of binding sites were present in NPC cultures (Kd = 3.01×10^{-9}/M and Kd = 1.25×10^{-10}/M) (Figure 4).
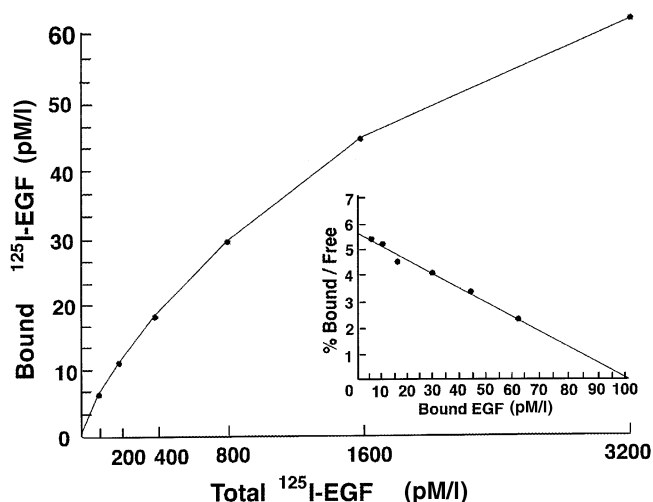
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Figure 3. Radio-ligand analysis of epidermal growth factor (EGF) binding sites in primary cytotrophoblast cell cultures. The main graph shows the saturation curve. A Scatchard plot is shown in the insert in which the data fit into a straight line. Each dot represents an average of triplicate cultures.
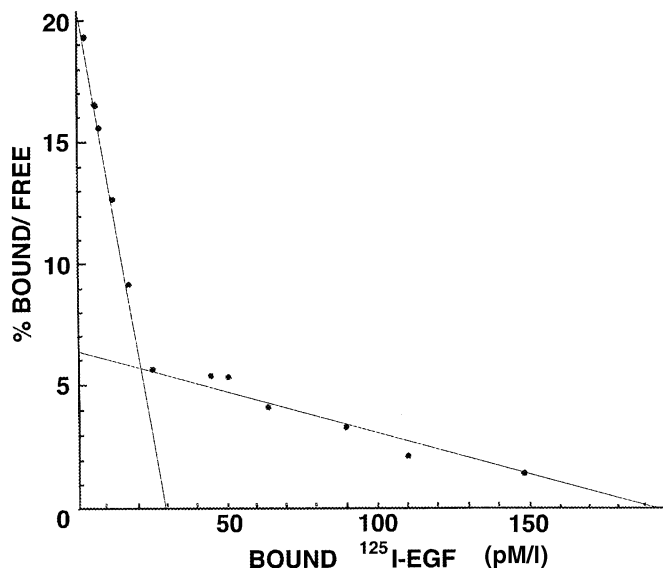

Figure 4. Scatchard plot of [^{125}I]-epidermal growth factor (EGF) binding in normal placental cytotrophoblast (NPC) cell cultures. Each dot represents an average of triplicate cultures. Note that the data fit into two straight lines.

Effect of IGF on NPC cell proliferation

Insulin is required for the proliferation of most cell types in serum-free culture. As expected, insulin caused stimulation of NPC cells. Insulin at a concentration of 3 µg/ml was sufficiently high to induce maximal stimulation, and there was no further increase in cell number at concentrations of insulin within 3–300 µg/ml range (Figure 5). The NPC cells also showed a concentration-dependent response to IGF-I (0.3–30 ng/ml) regardless of the presence or absence of EGF (Figure 6).

Effect of TGFβ on NPC cell proliferation

TGFβ1 reduced the level of stimulation of NPC cell proliferation induced by EGF. TGFβ1 at concentrations of 1–10 ng/ml resulted the reduction of EGF-stimulated cell proliferation by
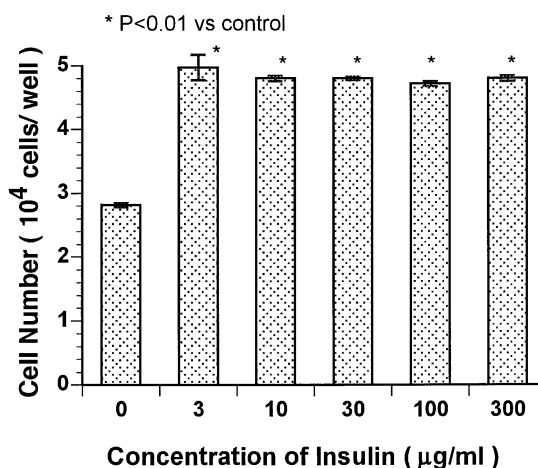
Figure 5. Effect of insulin upon normal placental cytotrophoblast (NPC) cell proliferation. NPC cells were seeded at 2×10^4 cells/well with epidermal growth factor (EGF) (10 ng/ml), 0.1% bovine serum albumin (BSA) and 10^{-6} M dexamethasone. Insulin was added to the cultures at different concentrations at plating. The cells were incubated for 48 h and counted with a Coulter counter after dissociation into single cells by trypsinization. Data are shown as mean ± SE of four replicate cultures and were tested for significance using Student's t-test.
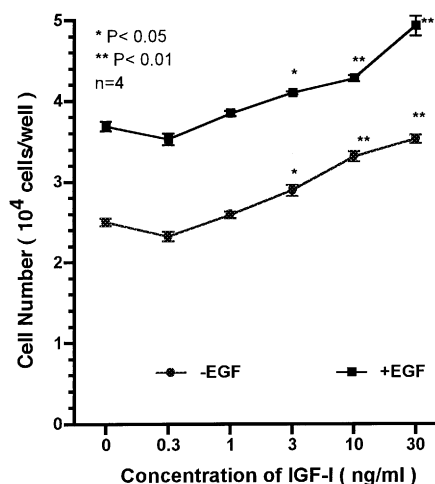

Figure 6. Effect of insulin-like growth factor I (IGF-I) on the proliferation of normal placental cytotrophoblast (NPC) cells. NPC cells were seeded at 2×10^4 cells/well with 0.1% bovine serum albumin (BSA), and 10^{-6} M dexamethasone in the presence or absence of epidermal growth factor (EGF) (10 ng/ml). IGF-I was added at different concentrations at plating. The cells were incubated for 48 h and counted with a Coulter counter after dissociation into single cells by trypsinization. Data are shown as the mean ± SE of four replicate cultures and were tested for significance using Student's t-test.

50% (Figure 7). This was due to inhibition of proliferation, rather than cell multinucleation, since the cells remained mononuclear after TGFβ1 treatment. Moreover, TGFβ1 by itself caused no inhibition in the absence of EGF (Figure 7).

Discussion

The placental cytotrophoblast cell line, NPC, was derived from human placental tissue obtained at the first trimester of gestational age. It had previously been characterized as pure
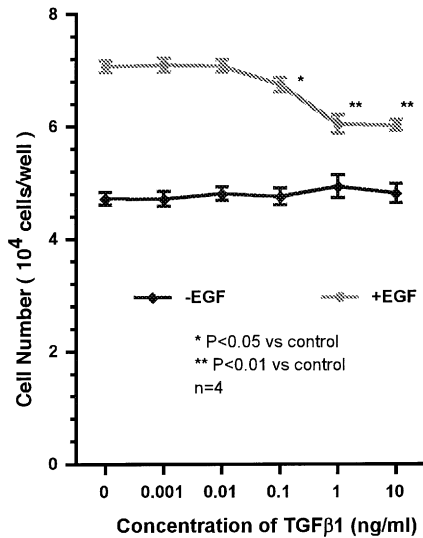
Figure 7. Effect of transforming growth factor-β1 (TGFβ1) on normal placental cytotrophoblast (NPC) cell proliferation. NPC cells were seeded at 2×10^4 cells/well with 0.1% bovine serum albumin (BSA), 10^{-6} M dexamethasone and epidermal growth factor (EGF) (10 ng/ml); 48 h after plating, the cells were washed twice with fresh FD medium and fresh medium was added with or without EGF. TGFβ1 was added to the cultures at different concentrations. The cells were incubated for a further 48 h and counted with a Coulter counter after dissociation into single cells by trypsinization. Data are shown as mean ± SE of four replicate cultures and were tested for significance using Student's *t*-test.

mononucleated, proliferating cytotrophoblast cells (Li *et al.*, 1996). The data obtained on this cell line therefore reflects the possible regulation of cytotrophoblast cell proliferation by growth factors *in vivo*. These growth factors that are synthesized by chorionic or decidual cells may regulate cytotrophoblast cell proliferation and differentiation in an autocrine/paracrine fashion.

EGF/TGFα may regulate cytotrophoblast cell proliferation in addition to their known role in regulating the differentiated functions of trophoblast cells. Both growth factors are polypeptides and stimulate cell replication in a variety of cell types through a common receptor. Our early report showed that EGF increased the production of HCG and steroid hormones in primary cultures of highly purified cytotrophoblast cells. It has also been shown that EGF increases HCG and steroid without affecting either cell number or DNA synthesis of transformed trophoblast cell lines and serum-containing trophoblast cell cultures (Bahn *et al.*, 1980; Huot *et al.*, 1981; Ilekis and Benaviste, 1985; Barnea *et al.*, 1990). Based on those observations, EGF was thought to be a differentiation factor for trophoblast cells. The present finding that EGF/TGFα stimulated NPC cell proliferation suggests that EGF/TGFα could be a potent mitogen for cytotrophoblast cells *in vivo*.

The role of EGF/TGFα in regulating cytotrophoblast cell proliferation is supported by demonstration of the presence of specific binding sites for these growth factors. The NPC cell line displayed two classes of EGF receptors, of high affinity and low affinity. However, only the low affinity binding sites were observed in non-proliferating cytotrophoblast cell primary

cultures as shown here and by others (Maruo *et al.*, 1987). Choriocarcinoma cells, upon which EGF did not show a growth promoting activity, have also been shown to possess only the low affinity binding site (Bahn *et al.* 1980). Taken together, the high affinity EGF receptor appears to be critical for the proliferative response of cytotrophoblast cells. This may reflect the in-vivo situation since different affinity EGF binding sites have been identified in chorionic villous tissue.

The regulation of cell proliferation and differentiated functions by EGF and TGFα in trophoblast cells are physiologically important. Although only a negligible amount of EGF (<0.01 ng/ml) has been detected in the circulation of human beings (Fisher and Lakshmanan, 1990), EGF and TGFα mRNA and the polypeptide products have been identified in both placental tissue and trophoblast cells (Hofmann *et al.*, 1991). Therefore, EGF and TGFα are more likely to be local regulators. In addition, immunohistochemical localization of EGF was reported in human endometrium and decidua (Hofmann *et al.*, 1991). EGF and TGFα mRNA have been shown to be expressed at high levels in mouse (Tamada *et al.*, 1991), rat (Han *et al.*, 1987) as well as human endometrium and decidua (Zhao *et al.*, 1992). Most of the proliferating cytotrophoblast cells are localized in the cytotrophoblast column and cytotrophoblast shell close to decidual tissues. Thus it is likely that the EGF and TGFα synthesized in decidual cells can easily diffuse into these structures and affect cytotrophoblast cell proliferation.

EGF and/or TGFα could be important mitogenic factors for cytotrophoblast cells; however, the proliferative effect of EGF/TGFα may be modulated by other growth factors, including members of the IGF family. IGFs may also regulate cytotrophoblast cell proliferation. IGF-I mRNA (Wang *et al.*, 1988) and its receptor (Czech *et al.* 1984) are expressed in cytotrophoblast cells, and IGF-I has been shown to stimulate NPC cell proliferation in the present paper. Moreover, IGF-II was shown to be expressed in proliferating cytotrophoblast cells (Shen *et al.*, 1986) and was shown to be able to bind IGF-I receptor and stimulate cell proliferation (Massague, 1982; Ohlsson *et al.*, 1989).

TGFβ is another growth factor which may regulate cytotrophoblast proliferation. This has been shown to possess proliferative activity in certain cell types but to inhibit the proliferation of other cell types. It has been shown to promote cell proliferation in mesenchymal cells but to induce differentiation in epithelial cells. In the mouse embryo, TGFβ1 expression is closely associated with the mesenchyme and is believed to be important for morphogenesis and angiogenesis. In human placenta, TGFβ1 gene was coexpressed with IGF-II and PDGF-B (Frolik *et al.*, 1983; Ohlsson, 1989). TGFβ was more likely to be a differentiation factor for trophoblast cells, since TGFβ1 consistently suppressed EGF-stimulated cell proliferation in NPC and in other trophoblast cell cultures (Graham *et al.*, 1992).

In summary, TGFα and/or EGF produced in the trophoblasts or in the decidua are probably the predominant mitogen for the replicating cytotrophoblast cells. This activity can be further enhanced or suppressed by other autocrine or paracrine

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mediators, including IGFs or TGFβ1. Meanwhile TGFβ1 is likely to be a differentiation factor for trophoblast cells.

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