Epidermal growth factor stimulates the cell growth of the PA-1 teratocarcinoma cell line in an autocrine/paracrine fashion

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In order to investigate the biological significance of epidermal growth factor (EGF) in the cell function of teratocarcinoma cells, we examined the production, binding and cell proliferative effect of EGF in PA-1 human ovarian teratocarcinoma cell line. The immunoreactivity of EGF in PA-1 cell-conditioned medium was detected by human EGF radioimmunoassay, and prepro-EGF mRNA was demonstrated in PA-1 cells by Northern blot analysis. An [125I] EGF binding study showed the presence of EGF receptor with very high binding affinity and relatively low numbers of binding sites in PA-1 cells. Furthermore, the growth of PA-1 cells was stimulated by EGF and inhibited by anti-EGF monoclonal antibody. These results suggest strongly that EGF plays an important role in controlling the growth of teratocarcinoma cells as an autocrine/paracrine growth factor.

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Teratocarcinomas are malignant gonadal tumors that contain an undifferentiated stem-cell population of embryonal carcinoma cells (EC cells), which are thought to be a good model system for investigating an early mammalian embryogenesis because they have many properties in common with the early normal embryonal cells (1–3). The PA-1 cell is a human ovarian teratocarcinoma cell line obtained from ascitic fluid cells of recurrent malignant ovarian teratoma (4) and is known to express some embryonic characteristics. In particular, late-passage PA-1 cells are known to resemble EC cells because they form embryoid bodies and express the PCC4 antigen characteristic of embryonic blastocysts (4), but they lack the F9 antigen characteristic of two-cell and more developed pre-implantation embryos. They are known also to be tumorigenic in nude mice and have activated ras oncogene (5).

Epidermal growth factor (EGF) is a polypeptide of approximately 6000 D and is known as a potent mitogen. The biological effect of EGF is exerted through binding to a specific 170,000-D glycoprotein receptor. In mouse EC cells, EGF receptor is reported to exist in cryptic, intracellular latent forms (6) and increases in number by differentiation of EC cells (7). Furthermore, low numbers of EGF receptors are expressed in human EC cells (8). Undifferentiated EC cells also are shown to contain the necessary components of protein tyrosine kinase signal transduction machinery (9). In spite of these many observations, there has been no report that indicates EGF secretion by EC cells, except Jing’s recent report that the production of EGF-like polypeptides by teratocarcinoma cells was shown in PA-1 cells by means of a bioassay for EGF (10).

In order to determine if EGF might play a significant role in the regulation of cell growth of teratocarcinoma cells through a possible autocrine/paracrine mechanism, we examined the production and secretion of EGF, EGF receptor and the cell proliferative effect of EGF action in teratocarcinoma cells using PA-1 cells. This study could contribute to the elucidation of the mechanism of EGF action in the control of early embryogenesis, not only because teratocarcinoma-derived EC stem-cell lines share the same biochemical and developmental properties as the early embryo but also because EC cells overcome the difficulties of dealing with small amounts of cells obtained from embryos of early stage, especially from preimplantation embryo.

Materials and methods

Cell culture

Late-passage (P334) PA-1 human ascitic fluid cells from an ovarian teratocarcinoma (ATCC CRL 1572) obtained from American Type Culture Collection (Rockville, MA) were grown routinely and maintained in minimum essential medium (MEM) (Nissui) supplemented with 10% fetal bovine serum (FBS) (Cell Culture Laboratories, Cleveland, OH) at 37°C in a humidified atmosphere of 95% air 5% CO₂. The medium was
changed every 2–3 days and cells were passaged every 5 days.

Epidermal growth factor radioimmunoassay for PA-1 cell-conditioned medium

In order to investigate whether immunoreactive EGF is secreted from cultured PA-1 cells, the concentration of human EGF (hEGF) in the medium conditioned by PA-1 cells was assayed by radioimmunoassay (RIA) for hEGF. The PA-1 cells were seeded in a 75-cm² tissue culture plate (Falcon, Lincoln Park, NJ) at the density of 2 × 10⁴ cells/cm² and cultured in MEM with 10% FBS for 4 days. After the medium was aspirated, cells were rinsed three times with phosphate-buffered saline (PBS) and cultured further in MEM without FBS for another day. This medium was collected as a conditioned medium, lyophilized and resuspended in appropriate amounts of PBS to carry out an RIA for hEGF. The RIA for hEGF was performed using recombinant hEGF (Wakunaga Pharmaceutical, Hiroshima, Japan), anti-hEGF antibody (Wakunaga Pharmaceutical, Hiroshima, Japan) and [125I] hEGF (Amasham Japan, Tokyo, Japan). In this RIA procedure, measurable EGF ranged from 40 to 1500 ng/l.

Ribonucleic acid preparation and Northern blot analysis of PA-1 cells

Ribonucleic acid (RNA) was isolated from PA-1 cells by Quick Prep mRNA Purification Kit (Pharmacia, Piscataway, NJ) after they were removed from culture flasks by a rubber policeman. Poly (A)⁺ selected RNA was electrophoresed in 1% agarose gels containing formaldehyde, followed by transfer to a nylon membrane. Blots then were baked at 80°C in a vacuum oven for 2 h. After prehybridization, blots were hybridized with 2 × 10⁶ cpm/ml of [32P] multiprime-labeled human prepro-EGF cDNA, which was kindly provided by Dr Graeme I Bell (11). Northern blot hybridization using this probe was carried out at 42°C overnight in 50% formamide, 4 × SSC (1 × SSC) is 0.15 mol/l NaCl plus 0.015 mol/l sodium citrate, 20 mmol/l TRIS · HCl (pH 7.0), 10 × Denhardt’s solution (20 × Denhardt’s solution is 0.4% each of bovine serum albumin, Ficoll and polyvinylpyrroldione), 0.1% SDS and 100 mg/l salmon sperm DNA. Washes were performed in 2 × SSC and 0.1% SDS at room temperature for 60 min and at 50°C for 20 min, and exposed to X-ray film (XAR-5, Kodak Co.) with an intensifying screen at −80°C.

The [125I] EGF binding study in PA-1 cells

After PA-1 cells were seeded on 35-mm 6-well plates (Falcon, Lincoln Park, NJ) at the density of 2 × 10⁴ cells/cm² and cultured for 4 days, they were rinsed with MEM supplemented with 10 mmol/l HEPES and 0.1% bovine serum albumin (BSA) and then incubated with MEM containing 6.7 × 10⁴ cpm of mouse [125I] EGF (NEN Research, Boston, MA., specific activity is 6700 kBq/μg) and various concentrations of unlabeled mouse EGF (Collaborative Research, Bedford, MA) for 2 h at room temperature. After aspirating the incubation mixture and rinsing the cells with PBS containing 0.1% BSA, cells were solubilized with 0.4 mol/l NaOH and bound radioactivity was measured in a gamma-counter.

Cell growth curve

The PA-1 cells were seeded on 24-well plates (Falcon, Lincoln Park, NJ) at 2 × 10⁴ cells/cm² and cultured in MEM containing 1% FBS, with or without 10 μg/l mouse EGF, for 14 days. Medium was changed every 2–3 days. Cell monolayers were detached from the plates with 0.1% trypsin and suspended into a single cell by pipetting, and the number of cells was counted in the hemocytometer.

[3H] Thymidine uptake of PA-1 cells

To examine the mechanism of EGF action on teratocarcinoma cells, the effects of EGF and anti-EGF antibody on [3H] thymidine incorporation were analyzed in PA-1 cells. The cells were seeded on 24-well plates at 5.6 × 10⁴ cells/cm² and cultured in MEM supplemented with 10% FBS for the first 3 days and in MEM without FBS for another 2 days. They were washed twice with PBS and replaced by MEM supplemented with various concentrations of hEGF or anti-hEGF monoclonal antibody (KME-10, Wakunaga Pharmaceuticals, Hiroshima, Japan) for 24 h. The cells were pulsed with [3H] thymidine (18.5 kBq) (NEN Research; specific activity is 74.0 GBq/mmol) in each well for the last 4 h of incubation.

Results

Human EGF RIA

As shown in Fig. 1 representatively, the serial dilution of conditioned medium showed a displacement curve of [125I] EGF binding that almost paralleled that of the hEGF standard in the hEGF RIA, suggesting that the conditioned medium contains a substance that is identical immunologically with hEGF. This assay revealed that the concentration of immunoreactive EGF in conditioned medium was 11.9 ± 0.57 pg/10⁶ cells per 24 h (mean ± SD, N = 4).

Northern blot analysis

As shown in Fig. 2. Northern blot hybridization using a human prepro-EGF cDNA probe revealed that a transcript of human prepro-EGF mRNA (about 5 kb
long) was detected clearly in cultured PA-1 cells, which was consistent with the size reported previously.

**The \[^{125}\text{I}]\text{EGF} binding study**

The representative Scatchard plot analysis of the \[^{125}\text{I}]\text{EGF} binding study of cultured PA-1 cells revealed that PA-1 cells possessed a specific EGF receptor with a curvilinear profile (Fig. 3). The dissociation constant and the number of receptors were \(2.22 \times 10^{-11}\) mol/l and \(1.20 \times 10^{3}\) cell in the high-affinity binding sites, respectively, and \(8.21 \times 10^{-10}\) mol/l and \(5.53 \times 10^{3}\) cell in the low-affinity binding sites, respectively.

**Cell growth curve**

As shown in Fig. 4, EGF stimulated significantly the growth of PA-1 cells from 5 days to 14 days after seeding. The EGF-treated cells grew and reached the plateau state more rapidly than the control.

**\[^{3}\text{H}]\text{Thymidine incorporation}**

Figures 5 and 6 show the effects of EGF and anti-EGF antibody on DNA synthesis in PA-1 cells. None of the different concentrations of EGF affected the incorporation of \[^{3}\text{H}]\text{thymidine} into PA-1 cells (Fig. 5). Anti-EGF monoclonal antibody, KME-10, reduced significantly the \[^{3}\text{H}]\text{thymidine} uptake at a concentration of 500 mg/l and there was no statistically significant difference in \[^{3}\text{H}]\text{thymidine} uptake between 0 mg/l and 500 mg/l mouse IgG (Fig. 6).

**Discussion**

Previous studies have established that EC cells not only produce growth factors such as platelet-derived growth factor (12–14), insulin-like growth factor (14–16), fibroblast growth factor (13, 17–19), transforming growth factors \(\alpha\) and \(\beta\) (14) and several incompletely characterized growth factors (17), but they possess also the receptors for growth factors (12, 16, 20, 21). In spite of these many reports about growth factors in EC cells, the production of EGF by teratocarcinoma cells has not been shown until the report of Jing et al. (10), which demonstrated the secretion of polypeptides related to EGF in PA-1 cells by means of a bioassay for EGF using the GH26H cell line and Western blot. So far, there has been no direct evidence for the production and secretion of EGF in PA-1 cells at a molecular level. Therefore, the results of the present study are the first to demonstrate that the PA-1 human teratocarcinoma cell line produces and secretes immunoreactive EGF, in view of the fact that prepro-EGF mRNA of about 5.0 kb was detected clearly by Northern blot hybridization and PA-1 cell-conditioned medium contained EGF immunoreactivity. Compared with the bioassay for EGF, the RIA for EGF provides a more specific determination of EGF activity in PA-1 cell-conditioned medium because the bioassay may contain other polypeptide growth factors related to EGF that respond positively to the bioassay for EGF. Considering the results of our experiments, EGF should be newly added to a group of several growth factors that were identified previously and characterized as being secreted by teratocarcinoma cells. While the presence of EGF receptor and its change during the differentiation of EC cells have been reported previously.
incorporation by PA-1 cells was inhibited by the addition of monoclonal anti-EGF antibody. From these results, it may be possible that the small amounts of EGF are sufficient to control the cell growth of the PA-1 cell through its receptor, which has a very high affinity for EGF, and that anti-EGF antibody has blocked the biological activities of endogenous EGF. Our result is contrary to the previous report of Jing et al. (10), which did not show the inhibitory effect of anti-EGF monoclonal antibody on DNA synthesis of PA-1 cells. The difference may be due to the experimental design, because Jing et al. used only small amounts of antibody
Fig. 5. Effect of epidermal growth factor (EGF) on $[^3$H] thymidine incorporation in PA-1 cells. The EGF was added to the cells in serum-free medium as described in the text. Each bar represents the standard deviation of quadruplicate measurements and experiments were repeated twice with similar results.

compared to our experiment. Relatively large amounts of anti-EGF antibody might be needed because the lowering of EGF in the medium might up-regulate the receptor population in addition to the very high affinity of EGF receptor to EGF. Concerning the effect of EGF on human EC cells, Verbeek et al. (23) similarly indicate autocrine secretion of EGF or EGF-like factor by human EC cells because they observed an inhibitory effect of monoclonal anti-EGF antibody on the $[^3$H]thymidine incorporation of the 2102EP human EC cell line. Overall, our results suggest strongly that EGF acts as an important growth stimulator of teratocarcinoma cells in an autocrine/paracrine manner. A similar mechanism of EGF action is reported in other human carcinomas; for example, Yoshida et al. (24) reported that EGF and/or TGF-α produced by carcinoma cells function as autocrine growth factors for human esophageal carcinomas. Embryonal carcinoma cell lines, which closely mimic important stages of early mammalian development, are widely recognized as a useful model system for a detailed understanding of the involvement of growth factors in the regulation of early mammalian embryogenesis. The experimental results obtained in our study on PA-1 cells indicate that EGF may be an important growth factor in controlling embryo development at the stage of the blastocyst.

Fig. 6. Effect of anti-epidermal growth factor (EGF) antibody on $[^3$H] thymidine incorporation in PA-1 cells. Anti-hEGF monoclonal antibody or control mouse IgG was added to the cells in serum-free medium as described in the text. Each bar represents the standard deviation of triplicate measurements and experiments were repeated twice with similar results.
because PA-1 cells reflect the characteristics of the blastocyst (4). More recently, evidence of the importance of some growth factors, including EGF, for the growth and development of preimplantation mouse embryos has been reported (25–32), suggesting the importance of EGF and other growth factors not only for the early fetal cells, like teratocarcinoma cells, but also for the preimplantation embryo itself.

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References


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