Divergent Effects of Epidermal Growth Factor and Transforming Growth Factors on a Human Endometrial Carcinoma Cell Line

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ABSTRACT

Epidermal growth factor (EGF), at concentrations ranging from 0.83 to 4.98 nM, markedly inhibited the proliferation of RL95-2 cells that were seeded at low plating densities (4.7 × 10^4 cells/cm²). Under the same incubation conditions, 16.6 pM EGF enhanced cell proliferation. At high plating densities (2.5 × 10^4 cells/cm²), 0.83 nM EGF also stimulated cell proliferation. Both the inhibitory and stimulatory effects of EGF were mimicked by transforming growth factor-α (TGF-α). However, the inhibitory action of TGF-α was always greater than that of EGF. Binding studies with [125I]-labeled TGF-α indicated that maximal cell surface binding of TGF-α occurred at 15 min, whereas maximal internalization occurred at 45 min. Both cell surface and internalized radioactivity declined sharply thereafter. Analysis of radioactivity released into the incubation medium during pulse-chase experiments indicated that RL95-2 cells extensively degraded both TGF-α and EGF. The lysosomotropic compound methylamine arrested the generation of low-molecular-weight degradation products of EGF, but not of TGF-α. In contrast to EGF and TGF-α, transforming growth factor-β (TGF-β) inhibited the proliferation of RL95-2 cells that were seeded at either low or high plating densities. Further, transforming growth factor-β induced the appearance of large cuboidal cells that were readily distinguished from cells treated with either EGF or TGF-α. These findings point to complex regulatory actions of growth factors on the proliferation of RL95-2 cells and suggest that the processing of TGF-α following EGF receptor activation is distinct from the processing of EGF.

INTRODUCTION

EGF is a heat-stable mitogenic polypeptide that participates in the regulation of a variety of cellular processes (1). The EGF receptor is a glycosylated phosphoprotein with intrinsic kinase activity that leads to autophosphorylation at tyrosine residues (2). Because tyrosine phosphorylation is also activated by other growth-promoting polypeptides (3) and by the products of certain oncogenes (4), it has been suggested that this reaction is important in the regulation of cell growth (5).

The effects of EGF may either be mimicked or modulated by transforming growth factors. These polypeptides have the ability to confer on certain normal cells a number of phenotypic changes that are usually associated with transformation (6, 7). TGF-α is structurally similar to EGF (8) and can bind and activate the EGF receptor (9, 10). Because there does not appear to be a distinct TGF-α receptor, it is generally accepted that the actions of TGF-α are mediated through the EGF receptor (11). In contrast to TGF-α, TGF-β binds to a distinct cell surface receptor and does not bind to the EGF receptor (12, 13).

We have recently reported that EGF inhibits the proliferation of RL95-2 human endometrial carcinoma cells when these cells are plated at low seeding densities (14). In the present study we sought to determine whether this effect is cell density dependent, and whether TGF-α mimics the effect of EGF on cell proliferation. In addition, we compared the effects of these growth factors with the actions of TGF-β. We now report that TGF-α exerts a more potent inhibitory effect on the proliferation of RL95-2 cells than EGF, and that the inhibitory actions of both EGF and TGF-α, but not TGF-β, are completely reversed at high plating densities.

MATERIALS AND METHODS

Cell Culture. RL95-2 cells were originally derived from an endometrial curettage of a poorly differentiated adenosquamous carcinoma of the endometrium (15). The present study was started after approximately 250 passages. Cells were grown in monolayer culture in 75-cm² tissue culture flasks, in DMEM supplemented with 2% FBS and antibiotics (100 units/ml penicillin; 100 μg/ml streptomycin). Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

For the individual experiments, rapidly growing cultures were harvested by rinsing the cells once with calcium- and magnesium-free Dulbecco’s phosphate-buffered saline, and once with 0.25% trypsin and 0.02% EDTA. Cells were then incubated for 5 min at 37°C. The cells were then resuspended in DMEM supplemented with 10% FBS to stop the action of trypsin. Cells were seeded in triplicate for each experimental condition, in either 12-well or 6-well Costar plates. Plating was done at the indicated densities in DMEM supplemented with 10% FBS. In all experiments cells were allowed to settle for 24 h. Medium was then changed to DMEM with 2% FBS, prior to making the indicated additions. Cell counts were performed with a hemocytometer because the growth factors altered the size and shape of RL95-2 cells. The intrassay variability of the cell counts was 2% or less.

Biologically active EGF was prepared from mouse submaxillary glands by the method of Savage and Cohen (16) and used in both biological activity and binding studies. Rat TGF-α (Peninsula Laboratories, Belmont, CA) and porcine TGF-β (R and D Systems, Minneapolis, MN) were used in biological activity studies. The batches of EGF and TGF-α used in the present study were equipotent in displacing 125I-EGF in a radioreceptor assay.

EGF Binding. To measure binding, mouse EGF and human TGF-α were iodinated by a modification of the chloramine-T method to a specific activity of 120 and 150 μCi/μg, respectively (17). The TGF-α was a gift from Dr. R. Derynck at Genentech (South San Francisco, CA). Human TGF-α was used in binding studies because attempts to iodinate rat TGF-β by the chloramine-T method were not successful. To perform binding studies, cells were grown in monolayer culture in either 35-mm or 22.6-mm multwell culture plates. Cells that were approximately 75% confluent were washed once in DMEM supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, containing 0.1% BSA (BM). Cells were then incubated in BM in the presence of labeled ligand. Nonspecific binding, determined in the presence of 1000-fold excess of unlabeled EGF, never exceeded 8% of total binding for either ligand. Incubations were stopped by washing cells 6 times in Hanks’ balanced salt solution containing 0.1% BSA. To monitor ligand internalization, cultured cells were washed as for binding studies and incubated for 4 min at 4°C with 500 mM NaCl that was titrated to pH 2.5 with acetic acid (18). Radioactivity removed by acid treatment (surface bound) and acid-resistant (internalized) radioactivity were then determined separately.
To study the degradation of radioatlabeled EGF and TGF-α, cells were labeled for 30 min at 37°C with the respective ligands. Cells then were washed twice in BM and resuspended in fresh BM supplemented with 200 ng/ml unlabeled EGF to prevent ligand rebinding. Dissociated radioactivity was analyzed by Sephadex G-25 column chromatography.

RESULTS

We have previously reported that the inhibitory effect of EGF on the proliferation of RL95-2 cells is gradually attenuated, but is not completely abolished, as the seeding density is increased from 2.5 to 17.5 × 10^3 cells/cm^2 (14). In 3 experiments (Fig. 1), EGF (0.83 nM) inhibited the proliferation of these cells by 32 ± 2% (P < 0.01) when seeded at low plating densities (4.7 × 10^3 cells/cm^2). However, at high seeding densities (2.5 × 10^4 cells/cm^2), the same concentration of EGF stimulated proliferation by 28 ± 4% (SE; P < 0.01). When the effects of various concentrations of EGF on cells plated at low seeding density were studied (Fig. 2), a very low concentration of EGF (16.6 pm) also enhanced cell proliferation. However, this stimulatory effect was significant only after 9 days from the initial plating of the cells, at which point the cell density in the control wells was approximately 1 × 10^6 cells/cm^2. In 3 experiments this stimulatory effect increased proliferation by 17 ± 3% (Table 1; P < 0.05). In the same series of experiments (Fig. 2), 0.83 nM EGF again inhibited cell proliferation. Higher concentrations of EGF (4.98 nM) did not exert an additional inhibitory effect.

TGF-α (0.83 nM) also inhibited the proliferation of RL95-2 cells that were seeded at a low plating density (Figs. 2 and 3; Table 1). The inhibitory effect of 0.83 nM TGF-α was significantly greater than that of a maximally inhibitory concentration of EGF (Table 1). In 4 experiments, 0.83 nM TGF-α inhibited cell proliferation by 90 ± 3% (P < 0.001). The concomitant addition of EGF and TGF-α did not produce a greater inhibitory effect than TGF-α alone (data not shown). In contrast to EGF, 16.6 pm TGF-α did not enhance cell growth (Table 1). However, 0.83 nM TGF-α enhanced cell growth when RL95-2 cells were seeded at high plating densities (data not shown). In three experiments, this concentration of TGF-α enhanced cell proliferation by 59 ± 22% (P < 0.05).

TGF-β inhibited the proliferation of RL95-2 cells that were plated at either low or high seeding densities (Fig. 3; Table 2). When seeded at low plating densities, a marked inhibitory effect was already evident at the lowest concentration tested in the present study (16.6 pm). Maximal inhibition occurred at a concentration of 0.22 nM TGF-β (Fig. 3). In three experiments, 0.22 nM TGF-β inhibited cell proliferation by 82 ± 2% (P < 0.005). This concentration of TGF-β exerted a similar effect on cells that were plated at high seeding density (Table 2), inhibiting proliferation by 75 ± 2% (P < 0.005).
REGULATION OF CELL PROLIFERATION BY GROWTH FACTORS

Endometrial carcinoma is an important health problem in Western societies (20). Recent epidemiological studies indicate that the incidence of this carcinoma has been increasing steadily during the past 20 yr, thereby superseding cervical cancer as the most common malignancy of the female genital tract (21). It has been estimated that in 1986 there were approximately 36,000 new cases of endometrial cancer, compared with 14,000 new cases of invasive cervical cancer (20). The reasons for this increasing incidence are not known. However, it is generally accepted that a number of risk factors may be contributing to make this cancer an increasingly serious health problem. These factors include infertility, obesity, dysfunctional uterine bleeding, failure of ovulation, prolonged estrogen therapy, improved nutrition, and an aging population (21). The mechanisms by which any of these risk factors may contribute to altering the propensity for malignant transformation of the normal endometrium are not known.

Several lines of evidence suggest that growth factors may participate in the regulation of endometrial proliferation. EGF receptors are present in normal human endometrium (22), human endometrial carcinoma cells (14), cultured guinea pig endometrial cells (23), and rat uterine membranes (24). EGF enhances the growth of cultured guinea pig endometrial cells (23). The mouse uterus contains EGF (25), and the sheep uterus contains a growth factor (26) that enhances the growth of rat uterine cells (24). Further, estrogen treatment results in increased levels of EGF in the mouse uterus (25) and increased EGF receptor number in rat uterus (27).

In the present study we have determined that the proliferation of RL95-2 endometrial carcinoma cells is modulated in a complex manner by two growth factors that activate the EGF

EGF, even at a concentration of 16.6 pM, tended to induce the appearance of fusiform cells. TGF-α exerted similar morphological changes, but also induced the appearance of giant cells (data not shown). Neither growth factor markedly altered the morphology of RL95-2 cells that were plated at high seeding density (Fig. 4). In contrast to EGF and TGF-α, TGF-β caused the cells to appear more cuboidal and induced the appearance of large polymorphic cells (Fig. 4). These effects were observed irrespective of the plating density. EGF did not completely reverse either the TGF-β-mediated changes (Fig. 4D) or the inhibitory action of TGF-β on cells plated at high seeding densities (data not shown). None of the growth factors altered the ability of the cells to exclude trypan blue.

We have previously reported that RL95-2 cells avidly bind and internalize 125I-EGF (14). We therefore sought to determine the characteristics of 125I-TGF-α binding in these cells. We first determined that unlabeled EGF and TGF-α, but not TGF-β, readily inhibited the binding of 125I-TGF-α (data not shown). As in the case of EGF, 125I-TGF-α readily bound to RL95-2 cells at 37°C (Fig. 5). The bound radioactivity distributed into a cell surface (acid-sensitive) compartment and an internalized (acid-resistant) compartment. Maximal cell surface binding occurred at 15 min and declined thereafter. In contrast, the amount of internalized 125I-TGF-α radioactivity continued to increase for 45 min and then declined sharply. To determine whether the decline in intracellular radioactivity was associated with degradation of the ligand, pulse-chase experiments were performed, and the radioactivity dissociating from the cells during the chase period was analyzed by Sephadex G-25 column chromatography (Fig. 6A). Unlabeled EGF was used during the chase period to prevent the rebinding of 125I-TGF-α that was released into the incubation medium (19). Ten min following the initiation of the chase, the majority of the radioactivity was in a high-molecular-weight peak migrating in the same region as intact TGF-α. However, at 180 min, the majority of the radioactivity was found in several low-molecular-weight peaks, as well as in a peak comigrating with free 125I. A similar pattern was observed with 125I-EGF (Fig. 7A).

When RL95-2 cells were preincubated with the lysosomotropic compound methylamine (10 mM) for 30 min prior to the addition of either 125I-TGF-α or 125I-EGF, there was a tendency for the amount of cell-associated radioactivity to increase (Table 3). Methylamine also decreased the amount of radioactivity released into the incubation medium during the subsequent 180-min chase period. This effect was always greater with 125I-EGF than with 125I-TGF-α (Table 3). Although methylamine did not alter the 125I-TGF-α postincubation profile (Fig. 6B), it completely blocked the appearance of low-molecular-weight peaks in the case of 125I-EGF (Fig. 7B).

DISCUSSION

Table 2  Effect of TGF-β on inhibition of cell proliferation

<table>
<thead>
<tr>
<th>Addition</th>
<th>Low seeding density (cell count x 10^5)</th>
<th>High seeding density (cell count x 10^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6.19 ± 0.60*</td>
<td>49.90 ± 4.70</td>
</tr>
<tr>
<td>TGF-β (166 pM)</td>
<td>1.01 ± 0.10</td>
<td>11.90 ± 0.30</td>
</tr>
</tbody>
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* Mean ± SD (n = 3) from a representative (of 3) experiment.

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Fig. 4. Morphological changes in RL95-2 cells that were seeded at high plating density. RL95-2 cells were seeded at 200,000 cells per 35-mm multiwell, and additions were made as indicated in the legend to Fig. 3. Cells were cultured for 6 days in the absence (A) or presence of 0.83 nM EGF (B), 0.22 nM TGF-β (C), and both 0.83 nM EGF and 0.22 nM TGF-β (D). All figures are phase-contrast micrographs of unstained RL95-2 cells at × 200 magnification.

Fig. 5. Time course of TGF-α binding and internalization. RL95-2 cells were incubated at 37°C with 1 ng/ml 125I-TGF-α. •, acid-sensitive radioactivity, as determined by elution into 0.5 M NaCl, titrated to pH 2.5 with acetic acid; ○, internalized radioactivity, as determined by solubilization of cells in 0.5 N NaOH following removal of surface-bound radioactivity. Points, mean (n = 3) from a representative (of 3) experiment; SDs were smaller than the size of the symbols.

Fig. 6. Elution profiles of 125I-TGF-α supernatant radioactivity. RL95-2 cells were incubated for 30 min at 37°C in the absence (A) or presence (B) of 10 mM methylamine, followed by the addition of 1 ng/ml 125I-TGF-α for 30 min. Cells were then washed twice and placed in medium supplemented with 200 ng/ml EGF for a second incubation period in the absence (A) or presence (B) of 10 mM methylamine. Medium was collected 10 min (●) and 180 min (○) later and analyzed by Sephadex G-25 chromatography.

receptor. Thus, both EGF and TGF-α inhibited the proliferation of cells that were plated at low seeding densities, but enhanced the proliferation of cells that were plated at high seeding densities. Although EGF is widely recognized as a mitogenic polypeptide, there are several other instances in which EGF has been reported to inhibit cell proliferation. Thus, EGF inhibits the growth of A431 human vulvar carcinoma cells (28, 29), certain human mammary (30) and squamous (31) carcinoma cells, a human hepatoma cell line (32), and GH4, as well as GH3D6, rat pituitary tumor cells (33, 34). The cell density dependence of the inhibitory effect of EGF in these cell lines is not known. However, EGF has been recently reported to inhibit the growth of R1E-1 rat intestinal cells that were plated at a low seeding density and to enhance their growth at a high seeding density (35). It has been suggested that the inhibitory action of EGF may be due to the presence of a large number of EGF receptors (36) or to EGF-induced changes in the cytoskeleton that interfere with the mitotic process (37). However, RL95-2 cells have only 150,000 surface EGF receptors per cell (14). Further, EGF can induce marked morphological changes in a clonal line of A431 cells without inhibiting the growth of these cells (38). It is likely, therefore, that EGF also acts via other mechanisms to inhibit cell proliferation.

The present findings are the first to indicate that TGF-α is a
were then washed twice and placed in medium supplemented with 200 ng/ml EGF for a second incubation period in the absence (A) or presence (B) of 10 µM methylamine, followed by the addition of 1 ng/ml 125I-EGF for 30 min. Cells were then incubated for 30 min at 37°C in the absence (A) or presence (H) of 10 µM methylamine. Medium was collected 10 min (■) and ISO min (○) later and analyzed by Sephadex G-25 chromatography.

Table 3 Effect of methylamine on the distribution of labeled ligands

<table>
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<tr>
<th>125I radioactivity (cpm)</th>
<th>No methylamine</th>
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<tr>
<td>Cell surface</td>
<td>249</td>
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<td>Intracellular</td>
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Fig. 7. Elution profiles of 125I-EGF supernatant radioactivity. RL95-2 cells were incubated for 30 min at 37°C in the absence (A) or presence (B) of 10 mM methylamine, followed by the addition of 1 ng/ml 125I-EGF for 30 min. Cells were then washed twice and placed in medium supplemented with 200 ng/ml EGF for a second incubation period in the absence (A) or presence (B) of 10 mM methylamine. Medium was collected 10 min (■) and ISO min (○) later and analyzed by Sephadex G-25 chromatography.

Table 3: Effect of methylamine on the distribution of labeled ligands

RL95-2 cells were incubated in 35-mm multiwell plates as in Fig. 8, in the presence or absence of methylamine, 125I-EGF (100,000 cpm/ml), and 125I-TGF-α (100,000 cpm/ml). Intracellular radioactivity was determined by solubilization of cells in 0.5 N NaOH following removal of surface-bound radioactivity with 0.5 M NaCl (pH 2.5). Values are means of duplicate determinations from a representative (of 3) experiments.

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Acknowledgments

We are grateful to Dr. John R. Davis for providing us with the RL95-2 cell line.

References


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