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EFFECTS OF TRANSFORMING GROWTH FACTORS AND REGULATION OF THEIR mRNA LEVELS IN TWO HUMAN ENDOMETRIAL ADENOCARCINOMA CELL LINES

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Summary—The effects of the transforming growth factor-β1 (TGF-β1) and epidermal growth factor (EGF) on the growth of cells from 2 endometrial cancer lines, Ishikawa and HEC-50 were evaluated by measuring rates of DNA synthesis and changes in cell numbers during culture. EGF at 17 and 1.7 nM concentrations consistently enhanced HEC-50 cell proliferation. TGF-β1 inhibited Ishikawa cell proliferation but, unexpectedly for epithelium-derived cells, stimulated HEC-50 cell growth. This effect is of interest as it indicates that endometrial cells can acquire an altered responsiveness to a growth inhibitor during the process of malignant transformation. Northern blot analyses showed expression of TGF-α, TGF-β1 and EGF receptors mRNA in both cell lines. Neither estradiol (E_2) nor 4-hydroxytamoxifen (OHTam) affected mRNA levels for either TGF-α or TGF-β in HEC-50 cells, a line unresponsive to E_2 for proliferation. In Ishikawa cells, previously shown to respond to both E_2 and OHTam by increasing proliferation rates, E_2 increased TGF-α mRNA and reduced TGF-β mRNA levels. OHTam lowered the levels of both mRNA species, although the effect was greater on TGF-β than TGF-α mRNA. These data are consistent with, but do not prove, the existence of a possible autocrine regulation by TGF-α and TGF-β of human cancer cell proliferation, which might be under E_2 influence in Ishikawa cells.

INTRODUCTION

Transforming growth factor-β (TGF-β), initially identified as a factor which can stimulate fibroblast growth in soft agar, is now known to affect many biological activities in a wide variety of tissues. The TGF-β family consists of 4 subtypes (TGF-β1–4) and distantly related homologs, such as activin, inhibin, and mullerian inhibitory substance (MIS). Receptors for TGF-β1 are present in most of the cell types tested but *in vitro* responses to this growth factor vary according to the cell type and culture conditions. For instance, TGF-β1 (the most widely studied factor in the TGF-β group) stimulates colony formation in soft agar by NRK cells, but it inhibits proliferation of the same cells in monolayer culture. For almost all epithelial cells, TGF-β1 is a potent negative regulator of proliferation, as described in several reviews [1–4]. It has been hypothesized that escape from growth inhibition by TGF-β is one of the important steps in carcinogenesis.

Epidermal growth factor (EGF) is an endogenous mitogen that also acts on a variety of different cell types, binding to a specific membrane receptor and promoting cell proliferation through tyrosine phosphorylation of specific proteins [5]. Transforming growth factor-α (TGF-α) has some structural homology with EGF, shares the same receptor and has equivalent biologic activities [1]. Both growth factors are considered to play important roles in the regulation of cancer cell proliferation.

In the studies reported here we tested the effects of TGF-β1 and EGF on DNA synthesis and cell proliferation in two human endometrial cancer lines, Ishikawa and HEC-50. We also evaluated the expression of mRNAs for these growth factors and for the EGF/TGF-α

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receptor, as well as the regulation by estradiol (E_2) and 4-hydroxytamoxifen (OHTam) of TGF-α mRNA and TGF-β mRNA levels in the 2 cell lines.

MATERIALS AND METHODS

Cells and culture conditions

The Ishikawa cell line originated from a well differentiated endometrial adenocarcinoma [6]. The HEC-50 line was established from cells collected from the ascites of a patient with poorly differentiated endometrial cancer [7].

Cells were maintained as described previously [8] and the experiments were carried out in a phenol red-free medium consisting of equal parts of Dulbecco MEM and Ham's F12 basal medium (BM), either in the presence or absence of 1% charcoal-treated fetal bovine serum (FBS), TGF-β1 or EGF (Collaborative Research, Bedford, MA).

[^{3}H]Thymidine incorporation assay

Cells were plated into 24 multiwell dishes at a concentration of 100,000 cells/well. One day after plating, medium in each dish was replaced by medium with the growth factors to be tested and cells were incubated for another 24 h. Incorporation of [^{3}H]thymidine was evaluated by a method described elsewhere [9], *viz.* exposing cells to media containing [^{3}H]thymidine (2 μCi/ml) for 1 h, adding ice-cold 10% trichloracetic acid (TCA), incubating in TCA at 0°C for 10 min, washing the precipitates twice with TCA, dissolving them in 0.25 N NaOH containing 40 μg/ml of DNA as carrier, and counting radioactivity in aliquots of the solutions with a scintillation spectrometer, using Liquiscint (National Diagnostics, Mansville, NJ) in the presence of 10% acetic acid.

Cell population growth

Cells were placed into 6 cm plastic dishes (Fisher) at a density of 500,000 cells/dish. One day after plating, the medium was replaced by BM + 1% FBS containing growth factors at various concentrations, renewed every other day. TGF-β1 was added immediately before use. Cell numbers on the day indicated in the figures were determined as average of 3 dishes, by using a hemocytometer.

Northern blot analysis

RNA was isolated by the guanidine thiocyanate/cesium chloride method [10] and enriched for poly (A+) RNA by one cycle of oligo (dT) cellulose chromatography [11]. Poly (A+) RNA, 10–15 μg, was denatured in 50% formamide and 2.2 M formaldehyde, size separated by electrophoresis on 1% (w/v) agarose gel containing 2.2 M formaldehyde and then blotted onto nitrocellulose. Filters were baked for 2 h at 80°C under vacuum, and then incubated in hybridization solution for at least 3 h. The filters were hybridized with the human TGF-α cDNA probe as described previously [12]. After the hybridization signal had decayed, the same filters were hybridized with a human TGF-β cDNA probe [13]. Hybridizations, usually for 24 h, were performed at 42°C in the presence of 50% (v/w) deionized formamide, 5 × Denhardt's solution (1 × Denhardt's, 0.02 v/w each of bovine serum albumin, Ficoll, and polyvinylpyrrolidine), 5 × SSPE (SSPE 1.15 M NaCl, 0.01 M NaH_2PO_4, 1 mM EDTA), 250 μg/ml denatured salmon sperm DNA and 0.1% sodium dodecyl

Table 1. Summary indicating the effects of EGF and TGF-β1 during 24 h incubations on [^{3}H]thymidine incorporation in HEC-50 and Ishikawa cells in each experiment

	Control	TGF-β1				EGF		
		1 nM	100 pM	10 pM	1 pM	17 nM	1.7 nM	0.17 nM
(A) [^{3}H]Thymidine incorporation in HEC-50 cells								
Exp.1H	1136 ± 239		1373 ± 359				2115 ± 423	
Exp.2H	2461 ± 86.7		3766 ± 205*			3915 ± 302*		
Exp.3H	3576 ± 139		5088 ± 208*			6466 ± 234*		
Exp.4H	6381 ± 132	9677 ± 456*	9586 ± 761*	8305 ± 612*	6299 ± 488	9896 ± 286*	9592 ± 294*	6923 ± 131*
Exp.5H	3383 ± 113	5617 ± 364*	4544 ± 78*	5525 ± 119*	3403 ± 193	6560 ± 343*	4030 ± 461	5525 ± 119*
(B) [^{3}H]Thymidine incorporation in Ishikawa cells								
Exp.1I	1806 ± 119		1384 ± 160*					
Exp.2I	9183 ± 1143		8897 ± 625	9097 ± 1410				
Exp.3I	4506 ± 351		3777 ± 71**					
Exp.4I	7602 ± 237		6548 ± 210*					
Exp.5I	7186 ± 560	5883 ± 487**	5866 ± 171*	6126 ± 283**	6970 ± 334			

The numbers shown are mean values of arbitrary counts of incorporated [^{3}H]thymidine in dpm and standard deviations. *$P < 0.01$, **$P < 0.05$.

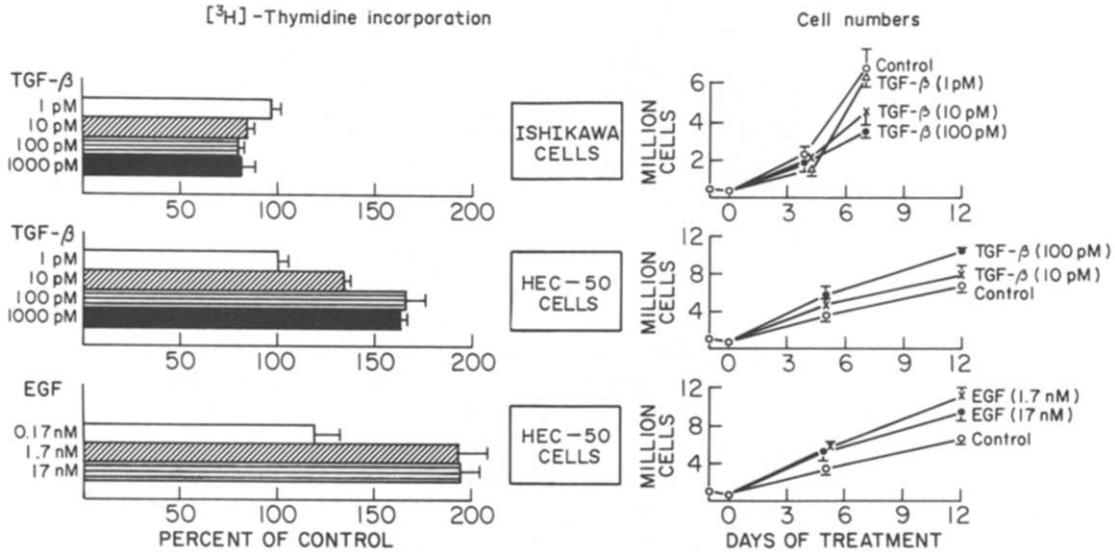

Fig. 1. The left panel shows the effects of EGF and TGF-β1 on [^{3}H]thymidine incorporation into HEC-50 and Ishikawa cells. Cells seeded in 24 multiwell plates at 100,000 cells/well density were exposed, 1 day after plating, to growth factors in BM + 1% FCS medium for 24 h. Subsequently, cells were incubated with media containing [^{3}H]thymidine for 1–3 h. Acid insoluble materials were counted in a scintillation spectrometer. Experiments were done in quadruplicate. Results were expressed as percentages of control values. Horizontal bars show standard deviations. The right panel shows growth curves of HEC-50 and Ishikawa cells cultured in BM + 1% FCS medium containing EGF and TGF-β1 at various concentrations. Cells in 1% ct-FBS BM (500,000/6 cm dish) were seeded in plastic dishes. Growth factors were added 24 h later (day 0) and media were changed every other day. Cell population size was determined by using a hemocytometer to count the number of cells present in 3 dishes on the days indicated in the figure. Vertical bars show standard deviation.

sulfate (SDS). At the end of the hybridization period the blots were washed twice in 2 × standard saline citrate (SSC), 0.1% SDS for 15–30 min at room temperature, followed by one wash in 0.1 ml SSC, 0.1% SDS for 15–30 min at 65°C. Filters were also hybridized

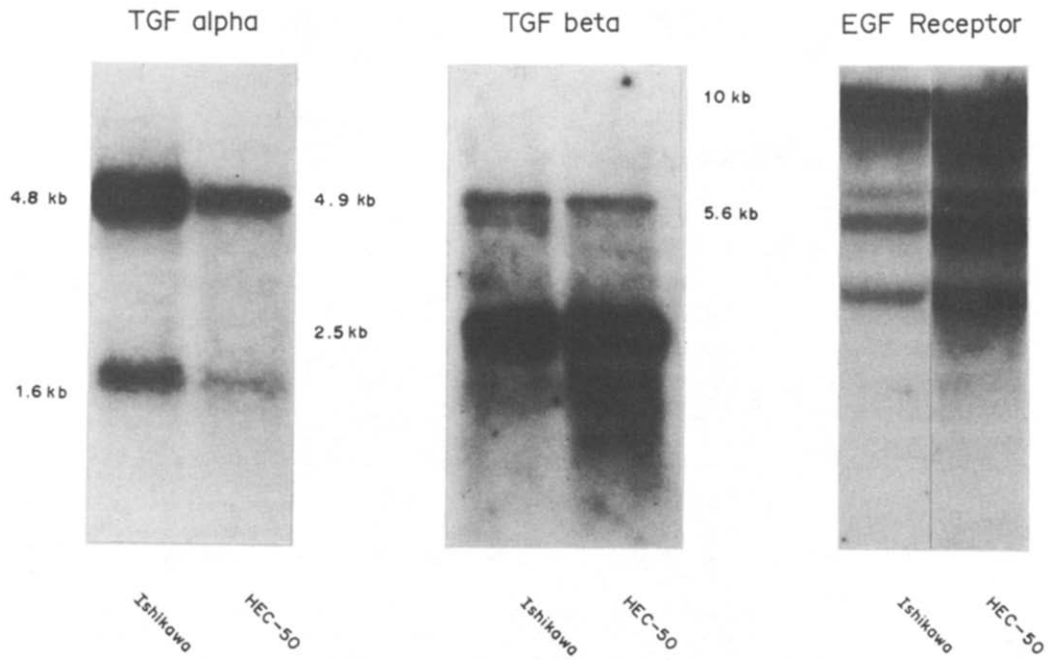

Fig. 2. Northern blot analyses show the expression of TGF-α, TGF-β and EGF-receptor in both Ishikawa Var-I and HEC-50 cells. Signals were obtained with 10 μg poly (A+) RNA isolated from Ishikawa and HEC-50 cells from subconfluent cultures and hybridized with human TGF-α, TGF-β and EGF-receptor cDNA probes.

with NB-29 [14], a cDNA which encodes a constitutively expressed heat shock-like protein. The signal obtained with this cDNA was used as a control for gel loading. Filters were exposed to Kodak XAR film at −70°C with an intensifying screen.

RESULTS

Table 1 shows effects of EGF and TGF-β1 at various concentrations on [^{3}H]thymidine incorporation from 4 experiments for each condition. EGF consistently stimulated DNA synthesis in HEC-50 cells. In 4 out of 5 experiments, TGF-β1 significantly inhibited DNA synthesis in Ishikawa cells and stimulated it in HEC-50 cells.

Figure 1, illustrating results from experiment 5H and 5I in Table 1, shows the effects of EGF and TGF-β1 on [^{3}H]thymidine incorporation and also presents growth curves for both Ishikawa and HEC-50 cells. Within the range of 10 pM to 1 nM, TGF-β1 significantly inhibited DNA synthesis in Ishikawa cells ($P < 0.05$). Maximum inhibition of DNA synthesis was observed at 100pM concentration. In the presence of 100pM TGF-β1 the numbers of Ishikawa cells were markedly reduced by day 7. In contrast, TGF-β1 concentrations ranging from 10 to 100pM significantly enhanced DNA synthesis in HEC-50 cells. Stimulation of DNA synthesis was concentration-dependent. As noted by days 5 and 12, TGF-β1 increased HEC-50 cell numbers, confirming the growth stimulatory actions observed in [^{3}H]thymidine incorporation experiments.

EGF also showed growth stimulatory effects in HEC-50 cells. At 1.7 and 17 nM concentrations, EGF almost doubled the rate of [^{3}H]thymidine incorporation in HEC-50 cells. At both concentrations, EGF increased by day 12 the numbers of cells to levels 2- to 3-fold higher than those of controls. The

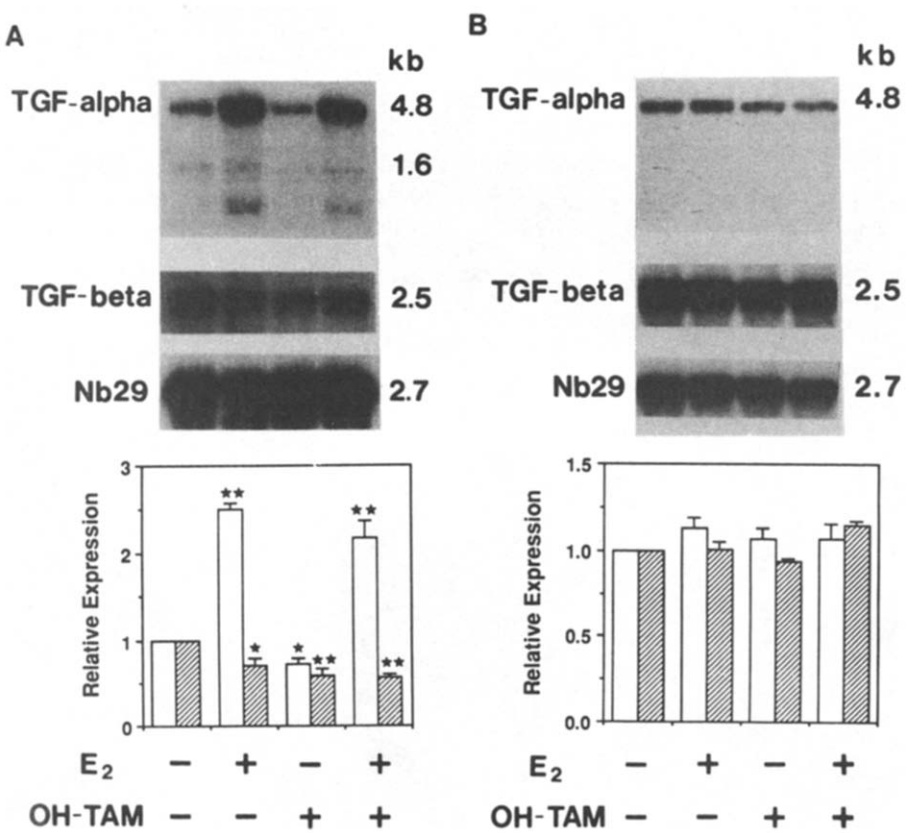

Fig. 3. The effects of estrogen and antiestrogen on accumulation of TGFs mRNA accumulation in Ishikawa and HEC-50 cells are shown in panels A and B, respectively. Cells were grown in basal medium containing 1% ctFBS with E_2 or OHTam or a combination of both compounds each at a final concentration of 100 nM for 24 h. Representative autoradiograms are shown in each panel. The hybridization signals obtained with the TGF-α cDNA (open bars histogram) and with the TGF-β1 cDNA (cross-hatched bars histogram) were quantified by densitometry and the mean ± SEM of 3 separate experiments for each condition are plotted in the lower panel as values relative to the untreated controls after correlation for gel loading. $^*P < 0.05$, $^{**}P < 0.01$ (treated vs control).

effect of EGF on Ishikawa cell growth was inconclusive in these experiments (data not shown).

Northern blot analysis demonstrated the presence of mRNA for TGF-α, TGF-β and EGF receptor in both cell lines (Fig. 2). TGF-α transcripts of 4.8 and 1.6 kb were identified in both Ishikawa and HEC 50 cells. These mRNAs were more abundant in Ishikawa than in HEC-50 cells. When the same nitrocellulose filter was rehybridized with TGF-β cDNA, an additional 2.5 kb mRNA was detected. The TGF-β mRNA was more abundant in HEC-50 cells. The abundance of the 10 and 5.6 kb EGF receptor mRNAs were similar in the two cell lines (Fig. 2).

In order to determine whether the expression of TGF-α could be influenced by E_2 and OHTam in Ishikawa and HEC-50 cells, these compounds were added to the culture media. When Ishikawa cells were incubated with E_2 at 100 nM concentration for 24 h, the TGF-α mRNA abundance relative to controls was increased approx. 3-fold [Fig. 3(A)]. This induction of TGF-α mRNA by E_2 was not reversed by equimolar concentrations of OHTam. In contrast to the effect of E_2, OHTam treatment resulted in a small but significant reduction in TGF-α mRNA levels; both E_2 and OHTam significantly reduced TGF-β1 mRNA abundance in Ishikawa cells.

Surprisingly, the lowering of TGF-α mRNA levels by OHTam was observed in culture conditions under which OHTam had been previously shown to stimulate cell proliferation [8]. It should be noted, however, that the reduction of TGF-β mRNA levels by OHTam is larger, resulting in a higher TGF-α/TGF-β mRNA ratio after exposure of Ishikawa cells to OHTam.

Neither E_2 nor OHTam had any significant effect on TGF-α or TGF-β mRNA levels in HEC-50 cells [Fig. 3(B)].

DISCUSSION

It has been suggested that growth factors mediate the effects of steroid hormones on the proliferation of breast and endometrial cancer cells [15]. In the present study we used two endometrial cancer cell lines, Ishikawa and HEC-50, to evaluate the effects of EGF and TGF-β1 on cell proliferation and the regulation of mRNA levels for these growth factors by E_2 and the antiestrogen OHTam.

Ishikawa cells are estrogen responsive endometrial cancer cell lines, and under our culture conditions, E_2 stimulated Ishikawa cell proliferation. In the present study, TGF-β1 significantly inhibited DNA synthesis and proliferation of Ishikawa cells as has been observed in other endometrial cancer cells lines [16]. Mullerian inhibitory substance, a member of the TGF-β family, was found to inhibit proliferation of HEC-1 endometrial cancer cells, but such an effect was not observed in other cancer cells tested in the same study [17].

When Ishikawa cells were treated with 100 nM E_2 or OHTam, the level of mRNA for TGF-β dropped significantly. Since this factor is growth inhibitory for these cells, and both E_2 and OHTam reduce its mRNA levels, it is possible that their effects on proliferation are mediated by TGF-β.

In contrast to the inhibitory action of TGF-β1 on Ishikawa cell proliferation, both TGF-β1 and EGF stimulated HEC-50 cell population growth. Growth stimulation of HEC-50 cells by TGF-β1 is of special interest because this factor is considered to be a potent growth inhibitor in most normal epithelial cells and carcinoma cells [18, 19], apparently acting as a negative autocrine regulator in these cells [20].

In many cases, TGF-β is secreted as a biologically inactive form, requiring post-translational modifications to become active [21, 22]. Some cancer cells, such as A549 human lung carcinoma, can grow in the presence of the latent form of TGF-β that they produce and secrete, whereas their proliferation is inhibited by exogenously added active TGF-β1 [23]. It has been hypothesized that uncontrolled growth of these cells might be partly due to an impaired TGF-β activation [23]. However, some cancer cells are also resistant to exogenously added TGF-β1 [24, 25], suggesting that other mechanisms are also involved. Boyd and Kaufman [24] have reported inhibitory effects of TGF-β1 in some of the various endometrial cancer cell lines tested and lack of responses in others [24]. The fact that HEC-50 cell growth can be stimulated by TGF-β1 suggests that through certain processes of malignant transformation some cancer cells not only escape from growth inhibition by the negative growth regulator but also acquire an altered responsiveness to this growth factor.

EGF has been shown to be involved in the regulation of human endometrial growth. The presence of the EGF receptor has been reported

in normal endometrial tissue [26] and in endometrial cancer cells [27]. EGF had a differential effect on proliferation in the RL95-2 human endometrial cancer line, depending on the density of cells initially plated [16]. Under our culture conditions, EGF markedly stimulated HEC-50 cell proliferation.

When assayed in cell culture systems, the activities of EGF and TGF-α were found to be virtually identical [1] and it is considered that TGF-α is the physiologic ligand for the EGF receptor in many cell types. These results would be compatible with an autocrine mechanism of TGF-α stimulation of HEC-50 cell proliferation. We have recently confirmed in another study that TGF-α does stimulate the proliferation of HEC-50 cells [28]. Neither E_2 (unpublished data) nor OHTam showed any significant effect on the growth of HEC-50 cells (unpublished observation) and neither TGF-α nor TGF-β expression were regulated by these compounds. The lack of responsiveness of HEC-50 to E_2 may result from alterations in the estrogen receptor [29].

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