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EFFECTS OF RETINOIC ACID ON ESTROGEN- AND THYROID HORMONE-INDUCED GROWTH IN A NEWLY ESTABLISHED RAT PITUITARY TUMOR CELL LINE

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Summary—In order to elucidate the complex mechanism(s) of action of steroid hormones, thyroid hormone and retinoic acid in pituitary mammotrophs, a clonal cell line (G3) was isolated from the rat pituitary tumor MtT/F84. G3 cells were found to secrete prolactin constitutively and to contain receptors for estrogen, glucocorticoid, progesterone and thyroid hormone. Stimulation of G3 cells with thyroid hormone resulted in a modest but significant increase in estrogen and progesterone receptor levels, however, retinoic acid treatment had no effect. Simultaneous addition of thyroid hormone and estrogen showed an additive effect on progesterone receptor levels in G3 cells. Thyroid hormone as well as estrogen enhanced the growth of G3 cells. Interestingly, retinoic acid was also found to enhance their growth but its enhancement was less potent than thyroid hormone and estrogen. Low concentrations of estradiol and thyroid hormone showed additive effects, but G3 cells stimulated with high concentrations of thyroid hormone failed to elicit an additive effect with estrogen, suggesting the presence of a common pathway in the growth-stimulatory actions of these hormones. In addition, exposure of G3 cells to retinoic acid completely abolished the effects of estrogen or thyroid hormone in terms of cell growth. These results suggest that there are complex interactions in the signalling pathways for estrogen, thyroid hormone and retinoic acid action in G3 cells.

INTRODUCTION

The thyroid hormone and retinoic acid (RA) receptor genes have been isolated and identified as related members of the steroid hormone receptor superfamily [1–3]. Mutational studies of the DNA-binding domain structure and target gene specificity have led to the classification of all the receptors and related proteins into either the glucocorticoid receptor (GR) or estrogen receptor (ER)/thyroid hormone receptor (TR) subfamily [4–6]. The RA receptors (RARs) have been classified as members of the ER/TR subfamily [7]. These members of the ER/TR subfamily display cross-recognition of hormone responsive elements (HREs) consisting of a core binding motif, TCAGGTCA, or a closely related sequence [8–10]. The biological effects of triiodothyronine (T_3), estradiol (E_2), and RA reveal that their cognate receptors can act to regulate distinct, but overlapping sets of genes. For example, TR and RAR can activate transcription from the rat growth hormone gene via a common HRE [11]. Functional antagonism among these nuclear receptors has also been reported [12]. These complicated interactions observed in the ER/TR subfamily can be at least partially explained by the recent findings that the precise spacing and the orientation of the core binding motif plays a critical role for recognition and activation of target genes by these receptors [13, 14].

E_2 and T_3 are well known regulators of various functions including the growth of target cells. Pituitary cells, particularly mammotrophs, are markedly affected by both E_2 and T_3 in terms of transformation and cell proliferation. Prolonged treatment of experimental animals with estrogen induces mammotroph hyperplasia and neoplasia [15]. Estrogen can stimulate growth of a transplantable prolactin secreting tumor *in vivo* [16, 17]. Thyroid hormone is also known to stimulate growth of cells derived from a pituitary tumor in culture [18–21]. However, the combined effects of estrogen and thyroid hormone on the growth of pituitary cells have not been fully elucidated. Recently, Ito *et al.* [22, 23] established a transplantable rat pituitary tumor

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(MtT/F84), the growth of which is stimulated by treatment with estrogen or thyroid hormone *in vivo*. They also found this tumor to be growth-stimulated by RA *in vivo* [24]. Therefore, this tumor appears to be an excellent model system to examine the combined effects of receptors from the ER/TR subfamily. To obtain more quantitative results on the effects of ligands acting via these receptors, we have established a cultured cell line from MtT/F84. The present study indicates that there are interesting agonistic or antagonistic interactions among these compounds in a newly established prolactin secreting tumor cell line.

EXPERIMENTAL

Chemicals

17β-[2,4,6,7-^{3}H]E_2 (95 Ci/mmol), [^{125}I]T_3 (315 Ci/mmol), [^{3}H]promegestone (R5020) (81.5 Ci/mmol), [1,2,4(n)-^{3}H]triamcinolone acetonide (41.8 Ci/mmol), [^{3}H]spiperone (22.5 Ci/mmol) and radioinert R5020 were purchased from DuPont/New England Nuclear Research Products (Boston, MA). Radioinert E_2, T_3, RA, dihydrotestosterone (DHT), dexamethasone (Dex), thyroxine (T_4), monoiodothyrosine (MIT), diiodothyronine (DIT), collagenase (type IV), trypsin and bovine serum albumin (BSA) (lyophilized and crystallized) were obtained from the Sigma Chemical Co. (St Louis, MO). Bromocriptine, 4-hydroxytamoxifen (OHTam) and, 1,25-dihydroxyvitamin D_3, RU486 and ORG2058 were kindly supplied by Sandoz Pharmaceuticals (Basal, Switzerland), ICI Pharm. (Osaka, Japan) and Dr M. Uskokovic (Hoffmann-La Roche Inc., Nutley, NJ), Roussel-Uclaf (Paris, France) and Radiochemical Centre Amersham (Bucks., England), respectively. Rat prolactin and anti-rat prolactin antibody were supplied from the National Pituitary Agency (NIDDK). The other reagents used were all of analytical grade.

Cell culture

The cell line (G3) was established from the transplantable rat pituitary tumor MtT/F84 [22]. MtT/F84 was removed from F344 rats (Charles River Japan Co. Ltd, Kanagawa, Japan), which had been treated with E_2 pellets. Primary culture and cloning were undertaken as described previously [25]. Briefly, the minced tumor was digested with 10 ml Hank's solution containing 0.1% (w/v) BSA, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4 at 20°C, 0.5 mg/ml collagenase and 0.1 mg/ml trypsin for 30 min at 37°C twice. Dispersed cells were cultured in Dulbecco's modified Eagle's medium (DME) containing 10% fetal calf serum (FCS) in 35-mm culture dishes in 95% air–5% CO_2 at 37°C. After 10- to 14-days of culture, transformed pituitary cells tended to clump together in small colonies. These cell clumps were removed to separate pituitary cells from contaminating mesenchymal cells, subjected to trypsinization [0.01% (w/v) trypsin–0.02% (w/v) EDTA in phosphate buffered saline (PBS)] and replated. Thereafter, the dishes underwent weekly passage. After three passages, the piled-up colonies were again removed. Subsequently, the cells were subcultured weekly. The cultured cells in 30th passage were cloned by limiting dilution. One clone, named G3, was maintained in DME containing 10% FCS. In the present study, the cells between the 80th and the 110th passages were employed.

MCF-7 cells, an estrogen-responsive human breast cancer cell line, were maintained in DME containing 10% FCS. GH_3 cells, a rat prolactin- and growth hormone-secreting pituitary tumor cell line, were maintained in DME–Ham's F-10 (1:1, v/v) containing 10% horse serum and 2.5% FCS.

Cell growth experiments

Exponentially growing G3 cells were harvested by trypsinization. Plastic culture dishes (35-mm) were each inoculated with 2×10^4 cells in 2 ml phenol red-free DME containing 5% dextran coated charcoal (DCC)-treated FCS. Treatment of FCS with DCC was shown to completely eliminate the endogenous E_2 and T_3 from FCS as assessed by measuring their concentrations in DCC-treated FCS by radioimmunoassay and counting the residual radioactivity in DCC-treated FCS after adding [^{3}H]E_2 or [^{125}I]T_3 to FCS. 24 h later (day 1), 2 μl aliquots of drug or vehicle (ethanol) were added directly to the culture medium, in which the final concentration of ethanol was 0.1%. This ethanol concentration was observed to be without effect on G3 cell growth. After the cells were cultured in 95% air–5% CO_2 and harvested with 0.01% trypsin–0.02% EDTA in PBS, number of viable cells counted on a hemocytometer by the trypan blue dye exclusion method on day 7, unless otherwise stated.

ER, progesterone receptor (PgR) and GR assays

To measure ER, PgR and GR levels, a whole cell binding assay was employed. G3 cells were plated into 24-well plates and grown to confluence. 24 h prior to assay, the medium was replaced with new medium supplemented with 2% DCC-treated FCS in the presence or absence of various test compounds. Monolayers were then washed twice with the binding buffer (DME containing 0.1% BSA) and incubated at 37°C in a final volume of 0.5 ml with 4 nM [^{3}H]$E_2 \pm 1\ \mu$M unlabeled E_2 for ER, 10 nM [^{3}H]R5020 $\pm$ 1 μM unlabeled R5020 for PgR, and 10 nM [^{3}H]triamcinolone actonide $\pm$ 1 μM unlabeled Dex for GR (single point saturation assays). Apparent equilibrium was obtained under these conditions after 1 h. Binding was terminated by placing the trays on ice, aspirating the supernatant, and washing the monolayer with ice-cold binding buffer four times. These cells were solubilized in 0.5 M NaOH–0.1% Triton X-100, and an aliquot was taken for estimation of the radioactivity. The maximum binding site and the dissociation constant were also determined in some experiments according to the method of Scatchard [26].

T_3 nuclear binding assay

This was performed according to the method of Burke and McGuire [27] with slight modification. G3 cells were plated into 60 mm-culture dishes and grown to confluence. 24 h prior to assay, the medium was replaced with DME supplemented with 2% DCC-treated FCS. Monolayers were washed twice with DME and incubated in DME with various concentrations (0.01–1 nM) of [^{125}I]T_3 in the presence or absence of unlabeled 100 nM T_3 for 90 min at 37°C. The cells were then scraped from the dishes in 0.5 ml of 20 mM Tris–HCl (pH 7.8 at 20°C), 1.1 mM $MgCl_2$, and 0.5% Triton X-100 and centrifuged for 10 min at 1000 ***g***. The supernatant was discarded and the nuclear pellet was resuspended in 1 ml of the buffer described above and centrifuged for 10 min at 1000 ***g***. After removal of the supernatant, the radioactivity in the nuclear pellet was determined.

Dopamine 2 (D2) receptor assay

D2 receptor was measured using a [^{3}H]spiperone binding assay as described previously [28].

Prolactin assay

Prolactin concentrations in conditioned media were measured by a double-antibody radioimmunoassay as described previously [28]. Intra- and inter-assay coefficients of variations were $<10\%$.

Statistics

The values presented in this paper are means $\pm$ SEM. The paired t test was used to test the differences between the means of experimental groups. P values of <0.05 were considered significant.

RESULTS

Presence of receptors for steroid hormones and T_3 in G3 cells

The first series of experiments were conducted to characterize the general nature of G3 cells. G3 cells were found to secrete prolactin (560 ng/10^6 cells/24 h) into the culture medium, suggesting that this newly established cell line exhibits the character of mammotrophs. This notion was further supported by the identification of D2 receptors in G3 cells (receptor number; 301,000/cell, K_d 3.8 nM). However, the secretion rate of prolactin was not significantly modulated by stimulation with E_2, T_3 or bromocriptine (data not shown). Steroid hormone receptors were measured by the Scatchard method and high-affinity binding sites for E_2, triamcinolone acetonide and R5020 were demonstrated (Table 1). These sites had the ligand specificity compatible with ER, GR and PgR, respectively [Fig. 1(A–C)]. The T_3 binding site was also examined as described in Experimental. Scatchard analysis showed that [^{125}I]T_3 binds to the nuclei of G3 cells with high affinity and low capacity (Table 1). The relative effectiveness of structural analogs related to T_3 revealed that the T_3 binding site identified in G3 cells is the TR [Fig. 1(D)]. Since RA is known to be associated with nonreceptor proteins [29] and cDNAs encoding rat RARs are not available, we did not analyze the RAR status of G3 cells.

Table 1. Concentration and ligand affinity of ER, PgR, GR and TR in G3 cells

	Sites per cell	K_d (nM)
ER	10,900 $\pm$ 569[a] (3)[b]	0.09 $\pm$ 0.006
PgR	17,400 (2)	2.8
GR	21,500 (2)	2.3
TR	8290 $\pm$ 631 (3)	0.085 $\pm$ 0.01

[a] Mean $\pm$ SEM.
[b] Number of experiments.

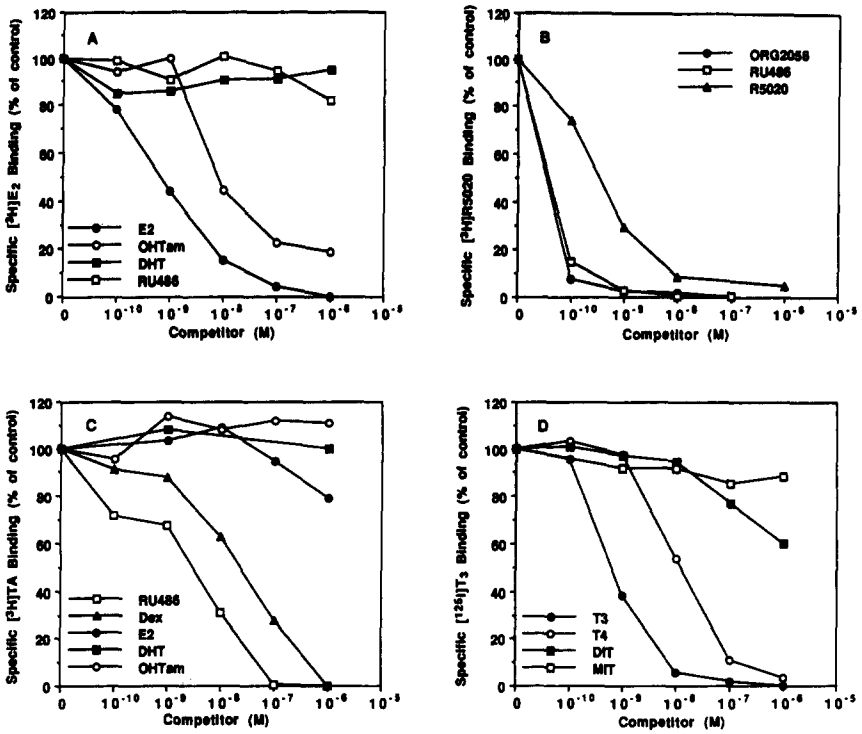

Fig. 1. Binding specificity of ER, PgR, GR and TR in G3 cells. Confluent cells were incubated with 4 nM [^{3}H]E_2 for ER (Panel A), 10 nM [^{3}H]R5020 for PgR (Panel B), 10 nM [^{3}H]triamcinolone acetonide (TA) for GR (Panel C) or 1 nM [^{125}I]T_3 for TR (Panel D) in the presence of various concentrations of competitors as described in Experimental. Binding is expressed as a percentage of that in control cultures in the absence of unlabeled ligand.

Hormonal regulation of these nuclear receptor levels was next addressed. Since PgR is a well-known marker for estrogen action in non-transformed as well as transformed estrogen target cells [30, 31]. PgR levels in hormone-treated G3 cells were analyzed. As shown in Table 2, the levels of PgR were up-regulated not only by E_2 but also by T_3 stimulation. The simultaneous addition of E_2 and T_3 resulted in further elevation of PgR levels. The stimulatory effect of T_3 on ER levels in G3 cells was also demonstrated (Table 2). These effects of E_2 and/or T_3 on PgR or ER were maximal after 24 h of stimulation. In addition, these increases in [^{3}H]R5020 and [^{3}H]E_2 binding with E_2 and/or T_3 were found to be due to increases in the number of binding sites without changes in their affinities (data not shown). On the other hand, Dex, testosterone, RA, and 1,25-dihydroxyvitamin D_3 failed to significantly change PgR or ER levels in G3 cells (Table 2). GR levels in G3 cells were not affected by any hormone treatment (data not shown).

Table 2. Effect of various hormones on PgR and ER levels in G3 cells

Treatment	PgR	ER
10^{-10} M T_3	111 ± 6.2[a]	131 ± 12.9
10^{-9} M T_3	136 ± 7.9[b]	176 ± 8.5[c]
10^{-8} M T_3	138 ± 9.3[b]	187 ± 1.9[c]
10^{-7} M T_3	137 ± 1.3[c]	191 ± 7.2[c]
10^{-8} M E_2	166 ± 11.4[c]	ND[f]
10^{-8} M $E_2 + 10^{-10}$ M T_3	173 ± 26.3[b]	ND
10^{-8} M $E_2 + 10^{-9}$ M T_3	211 ± 18.1[c]	ND
10^{-8} M $E_2 + 10^{-8}$ M T_3	267 ± 26.0[c,d]	ND
10^{-8} M $E_2 + 10^{-7}$ M T_3	246 ± 16.6[c,d]	ND
10^{-8} M Dex	92 ± 8.9	98 ± 6.9
10^{-8} M Testosterone	113 ± 9.8	95 ± 4.7
10^{-8} M RA	100 ± 5.4	103 ± 5.1
10^{-8} M 1,25-dihydroxyvitamin D_3	92 ± 6.8	108 ± 9.9

[a] Mean ± SEM of 3 or 4 determinations. Data are expressed as a percentage of specific binding in untreated cells.
[b] $P < 0.05$ vs control cells.
[c] $P < 0.01$ vs control cells.
[d] $P < 0.05$ vs cells treated with E_2 alone.
[e] $P < 0.001$ vs control cells.
[f] Not done.

Growth-stimulatory effects of E_2, T_3 or RA on G3 cells

G3 cells were exposed to each compound for 6 days and the number of viable cells counted. As shown in Fig. 2(A), the proliferation of G3 cells was stimulated by E_2 in a concentration-dependent manner. This growth-stimulatory effect of low concentrations (10^{-11}–10^{-10} M) of E_2 was completely inhibited by simultaneous addition of 10^{-8} M OHTam. The effect of higher concentrations (10^{-8} M) of E_2 was also inhibited by 10^{-7} M of OHTam (data not

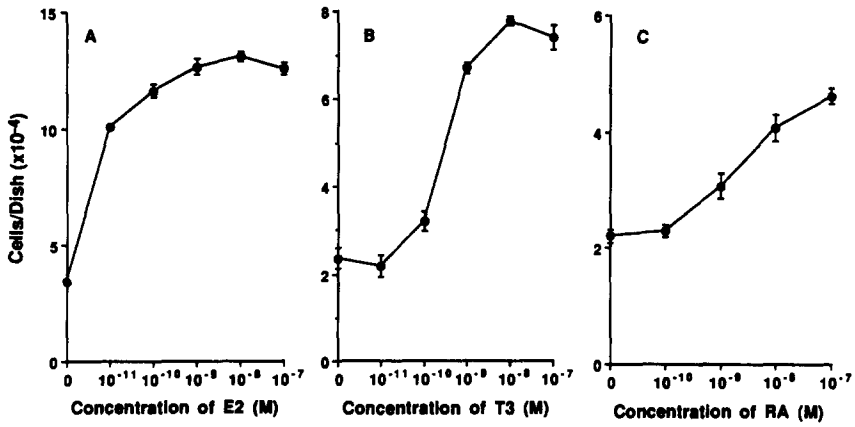

Fig. 2. Stimulatory effects of E_2, T_3 and RA on G3 cell growth. G3 cells (2×10^4 cells/dish) were plated and cultured with various concentrations of E_2 (Panel A), T_3 (Panel B) or RA (Panel C). On day 7, cell numbers were estimated. Data are the mean ± SEM (bars) of 3 determinations. Where error bars are not shown, they do not exceed the size of the symbol.

shown). T_3 also exhibited growth-stimulatory activity [Fig. 2(B)]. When RA was added, the proliferation of G3 cells was significantly enhanced at concentrations $> 10^{-9}$ M [Fig. 2(C)]. However, the ability of RA to stimulate the growth of G3 cells was found to be somehow less than that of E_2 or T_3 (also see below). Doubling times, calculated from the exponential phase of the growth curves, were 11.4 ± 3.9 ($n = 5$), 2.84 ± 0.15 ($n = 4$, $P < 0.05$ vs control), 2.47 ± 0.26 ($n = 4$, $P < 0.05$ vs control cells) and 4.76 ± 0.44 days ($n = 4$, $P < 0.05$ vs control cells) for control, 10^{-8} M E_2, 10^{-8} M T_3 and 10^{-7} M RA treated cells, respectively.

Effects of E_2 on the proliferation of T_3-stimulated cells

Next, we examined the effect of E_2 on the growth rate of T_3-stimulated G3 cells. Stimulation of G3 cells with both E_2 and T_3 exhibited a clear additive effect at low concentrations (Fig. 3). When G3 cells were exposed to higher concentrations (10^{-9}–10^{-8} M) of T_3, the additive effects of E_2 were markedly attenuated. This was not due to the fact that the high concentrations of T_3 maximally stimulated the growth of G3 cells, since 10% FCS exhibited a more profound effect on G3 cell growth than did 10^{-8} M T_3 (data not shown).

Effect of RA on the growth of E_2- or T_3-stimulated G3 cells

RA alone enhanced the proliferation of G3 cells [Fig. 4, also see Fig. 2(C)]. In the absence of RA, E_2 stimulated cell proliferation in a dose-dependent manner as stated above. However, addition of 10^{-7} M RA markedly attenuated the E_2-induced increase in cell yield, resulting in the complete abolition of E_2 responsiveness. This blunting of the E_2 effect by RA treatment was not due to a change in ER content since RA treatment did not cause a decrease in ER content (see Table 2). A similar effect of RA was also observed in T_3-treated cells. The simultaneous addition of 10^{-7} M RA completely abolished the T_3 effect on cell growth [Fig. 4(B)]. These effects of RA on E_2- or T_3-enhanced cell growth were observed at relatively low concentrations of RA (10^{-8}–10^{-7} M).

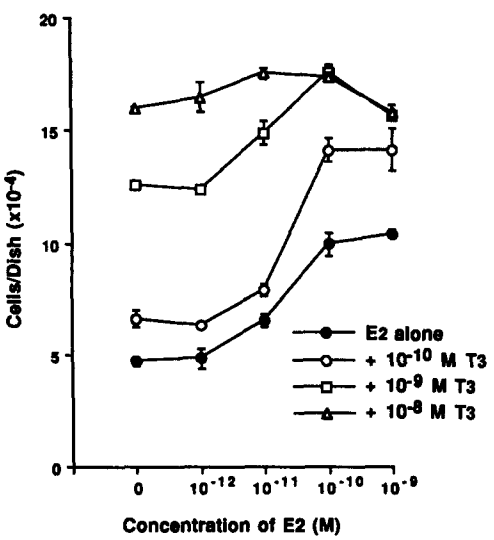

Fig. 3. Combined effects of E_2 and T_3 on G3 cell growth. G3 cells (2×10^4 cells/dish) were plated and cultured in the presence of various concentrations of E_2 alone or with T_3. On day 7, cell numbers were estimated. Data are the mean ± SEM (bars) of 3 determinations. Where error bars are not shown, they did not exceed the size of the symbol.

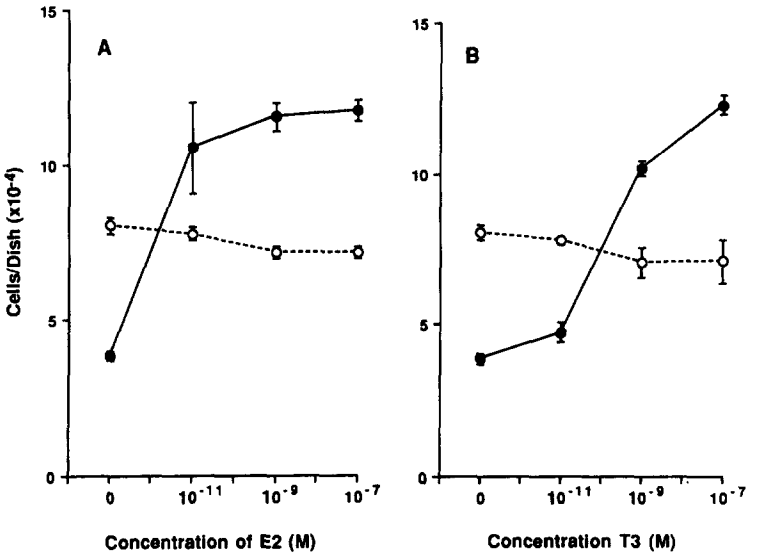

Fig. 4. Antagonistic effect of RA on E_2- and T_3-induced G3 cell growth. G3 cells (2×10^4 cells/dish) were plated and cultured with various concentrations of E_2 (Panel A) or T_3 (Panel B) in the presence (open symbols) or absence (closed symbols) of 10^{-7} M RA. On day 7, the cell numbers were recorded. Data are the means ± SEM (bars) of 3 determinations.

DISCUSSION

In the present study, we have used a newly established transformed prolactin secreting cell line (G3) to investigate the complex mechanism(s) of action of steroid hormones, thyroid hormone and RA. G3 cells contain various nuclear receptors whose levels are interestingly controlled by the hormonal conditions. In relation to the regulation of PgR levels, the expression of this receptor has been reported to be regulated by E_2 [30, 31], progesterone [32], and RA [33]. The present finding demonstrates that PgR levels are also up-regulated by T_3 as well as E_2 in G3 cells. This effect of T_3 on PgR levels may be cell-type specific, since T_3 also up-regulated PgR levels in GH_3 cells but not in MCF-7 cells (data not shown). The increase in ER levels following T_3 treatment is consistent with the reported findings on the *in vivo* studies using MtT/F84 tumor [23] as well as normal anterior pituitary gland [34, 35]. Therefore, the additive effect of E_2 and T_3 on PgR levels identified in this study may be explained by T_3-induced up-regulation of ER, although the possibility that both hormones possess independent signalling pathways for PgR regulation can not be eliminated. In any event, these increases of ER and PgR levels by T_3 are unlikely to be a consequence of a general increase in protein synthesis in response to T_3, since GR levels in G3 cells were not affected by T_3.

The most noticeable observation in this communication is the interactions of compounds which activate the ER/TR subclass of nuclear receptors. All three ligands (E_2, T_3 and RA) were able to stimulate G3 cell growth. E_2 and T_3 showed some additive effects at the low concentrations but when G3 cells were stimulated by high concentrations of T_3, E_2 failed to enhance the growth of G3 cells further. This suggests that both ligands share a common signalling pathway in expressing their growth-promoting ability in this cell type. This suggestion is supported by the finding that RA antagonized both E_2- and T_3-induced enhancement of G3 cell growth. These results are in contrast to those on the regulation of the PgR levels by E_2 and T_3. It is of interest that RA exhibits agonist action on unstimulated G3 cells but antagonist effects on G3 cells stimulated with E_2 or T_3. Although RA is known to stimulate growth hormone secretion and growth hormone mRNA production [11, 36], the effect of RA on pituitary tumor cell growth is largely unknown. To our knowledge, antagonist effects of RA on E_2- or T_3-induced cell growth have not been reported previously. However, it is difficult to explain these interesting events at the molecular level, particularly because many unknown gene products may be involved in the regulation of G3 cell proliferation. In this regard, the report that thyroid hormone induces an autocrine growth factor in pituitary tumor cells [21] suggests that there are key gene product(s) involved in growth

control that are induced by these hormones. Since RA-induced formation of the heterodimer between RAR and other nuclear receptor might occur [37], the heterodimeric nuclear receptors may activate similar genes present in G3 cells. However, the lack of RA effects on PgR or ER levels suggests that heterodimer formation between RAR and TR or ER may not play a role in the regulation of G3 cell functions. Another possibility is that ER, TR and RAR activate transcription of the key growth-promoting gene(s) via an overlapping responsive element, since T_3, RA and E_2 responsive elements have conserved DNA half-sites (core binding motif, TCAGGTCA). RAR binding to RA responsive elements may block the binding of ER or TR to their cognate responsive elements, where core binding motifs for RA responsive element (possibly arranged in direct repeat [13]) are shared with estrogen or T_3 responsive element. This possibility cannot be directly addressed at present since the gene(s) mediating E_2- or T_3-induced cell growth are yet to be isolated.

We previously reported that RA does not have antagonistic effects on E_2-induced cell proliferation in the human breast cancer cells T-47D, which are growth-inhibited by RA alone [38]. This is not surprising since the cell-type specific mechanisms of action of RA are well known [39].

The clinical significance of the present findings should be considered. Prolactin secreting adenomas can be treated with anti-estrogens [40, 41], however, thyroidectomy cannot be carried out since T_3 is a prerequisite for human life. The present finding that RA can antagonize T_3-induced enhancement of cell growth raises the possibility of RA treatment. To validate this possibility, RA effects on E_2- and/or T_3-responsive prolactin-secreting adenomas should be systematically examined.

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