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RETINOIC ACID ACTS SYNERGISTICALLY WITH 1,25-DIHYDROXYVITAMIN D_3 OR ANTIOESTROGEN TO INHIBIT T-47D HUMAN BREAST CANCER CELL PROLIFERATION

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Summary—Although retinoic acid has been shown to inhibit proliferation in human breast cancer cells, the mechanisms by which these effects are mediated are not known. Since several steroid hormones and their synthetic antagonists also inhibit proliferation of human breast cancer cells, we investigated the interactions between retinoic acid, 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] and antioestrogens in the control of human breast cancer cell proliferation *in vitro*. When T-47D cells, the most sensitive of six human breast cancer cell lines to the growth inhibitory effects of retinoic acid, were treated with retinoic acid and 1,25-$(OH)_2D_3$, a synergistic inhibitory effect on cell growth was observed. Retinoic acid also enhanced the growth inhibitory effect of various antioestrogens (4-hydroxytamoxifen, 4-hydroxyclomiphene or LY117018). However, retinoic acid did not affect oestradiol-induced growth stimulation. Measurement of the cellular receptors for 1,25-$(OH)_2D_3$ and oestrogen revealed no significant change in receptor levels following treatment with concentrations of retinoic acid which modulated growth.

These results indicate that retinoic acid not only has direct growth inhibitory effects on breast cancer cell proliferation but also augments the effects of some other known regulators of breast cancer cell replication including 1,25-$(OH)_2D_3$ and antioestrogens. Synergism appears to involve interactions with steroid hormone action distinct from changes in steroid hormone receptor levels.

INTRODUCTION

Retinoic acid (RA) and its analogues (retinoids) play an important role in the maintenance of normal growth and in the differentiation of epithelial tissues [1, 2]. Retinoids have also been shown to inhibit the growth of several different tumour cell lines. In addition, retinoids can induce the differentiation of embryonal carcinoma cell lines [3]. Several recent studies have examined possible interactions between retinoids and mammary tumours. Retinoids have been shown to inhibit rat mammary carcinogenesis induced by 7,12-dimethylbenz(*a*)anthracene and by *N*-methyl-*N*-nitrosourea [4, 5], and decrease the initial tumour growth rate of transplantable mammary adenocarcinoma in mice [6]. Furthermore, retinoids have been shown to have growth inhibitory effects on several rat, murine and human breast cancer cell lines *in vitro* [7, 8].

The growth of hormone-dependent breast cancer cells is also specifically inhibited by synthetic antioestrogens acting predominantly via the oestrogen receptor (ER) [9, 10]. Recently 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] also has been shown to have inhibitory effects on the replication of some human breast cancer cell lines *in vitro* [11, 12] and other tumours *in vivo* [13].

The present study was undertaken to assess the combined influence of RA and 1,25-$(OH)_2D_3$ or oestrogen/antioestrogen on a hormone-responsive human breast cancer cell line, T-47D, *in vitro* in order to assess if common mechanisms might be involved.

EXPERIMENTAL

Materials

1,25-Dihydroxy[26,27 methyl-^{3}H]vitamin D_3 (SA 176 Ci/mmol) and 17β-[2,4,6,7,16,17-^{3}H]-oestradiol (E_2) (SA 140 Ci/mmol) were obtained

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from Amersham, Australia (Sydney, Australia). All-*trans* RA and E_2 were purchased from Sigma Chemical Co. (St Louis, MO). 1,25-$(OH)_2D_3$ was the generous gift of Dr M. Uskokovic (Hoffmann-La Roche Inc., Nutley, NJ) to Dr John Eisman, Garvan Institute. 4-Hydroxytamoxifen (OHTam), *trans*-1-(4β-dimethylamino-ethoxyphenyl)-1-(4-hydroxy-phenyl)-2-phenylbut-1-ene, was supplied by ICI Pharmaceuticals Division (Macclesfield, U.K.) through the courtesy of Dr Alan Wakeling. 4-Hydroxyclomiphene (OHClom), 1-(4-β-dimethylaminoethoxy-phenyl)-1-(4-hydroxyphenyl)-2-chloro-2-phenylethylene, was synthesized as described previously [14] and generously donated by Dr Peter Ruenitz (College of Pharmacy, Athens, GA). LY117018,6-hydroxy-2-(*p*-hydroxyphenyl)-benzo(*b*)-thien-3-yl-*p*-2-(pyrrolidinyl)ethoxy phenyl ketone, was obtained from Lilly Industries Pty Ltd. (West Ryde, NSW, Australia). Porcine insulin (Actrapid) was obtained from CSL-Novo Industries (Sydney, Australia) and stored at 4°C. Other tissue culture materials were purchased from Flow Laboratories (Sydney, Australia).

Antioestrogens, E_2, RA and 1,25-$(OH)_2D_3$ were prepared as 1000-fold concentrated stock solutions in analytical reagent grade ethanol and stored at −20°C.

Cell culture

MCF-7 cells were supplied by Dr C. McGrath, Meyer L Prentis Cancer Center (Detroit, MI), while T-47D, ZR-75-1, BT-20, MDA-MB-231 and MCF-7M (a subline of MCF-7) cells were provided by E. G. and G. Mason Research Institute (Worcester, MA) for the National Cancer Institute Breast Cancer Program Cell Bank. Cells were maintained in RPMI 1640 medium supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid buffer, 14 mM sodium bicarbonate, 6 mM L-glutamine, 20 μg/ml gentamicin, 10 μg/ml porcine insulin, 6 μg/ml phenol red and 10% foetal calf serum (FCS) as described previously [15]. Modifications to this base medium were made in some experiments and these are identified in the figure legends.

Cell growth experiments

The experimental design consisted of plating 5×10^4 exponentially growing cells into 25-cm^2 flasks in 5 ml of RPMI 1640 medium with the concentration of FCS reduced to 5%, except where noted in the text. When cell numbers had doubled to approx. 10^5 cells/flask, 5 μl aliquots of drug or vehicle (ethanol) were added directly to the culture medium. At various times thereafter cells were harvested with 0.05% trypsin: 0.02% EDTA in Ca^{2+}-Mg^{2+}-free phosphate buffered saline and cell number was determined using a Coulter Counter. For evaluation of the data, the definition of "synergism" in this report is according to the following formula [16].

$$\frac{Ac}{Ae} + \frac{Bc}{Be} < 1,$$

where the doses of A or B alone that produce some growth-inhibitory effect are Ae and Be, and their doses in a combination that also has this same inhibitory effect are Ac and Bc.

ER and 1,25-$(OH)_2D_3$ receptor assays

For ER and 1,25-$(OH)_2D_3$ receptor studies, a whole cell binding assay was employed as described previously [17, 18]. Cells were placed into 24-well tissue culture trays and grown to confluence. Twenty-four hours prior to assay, medium was removed and replaced with new medium supplemented with 1% dextran coated charcoal treated FCS [17] and various concentrations of RA in ethanol, to give a final ethanol concentration of 0.1%. The incubation was continued at 37°C for 24 h. Monolayers were then washed twice with binding buffer [RPMI 1640 medium supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid, pH 7.4 and 0.1% bovine serum albumin (BSA)] and incubated at 37°C in a final vol of 0.5 ml with 4 nM [^{3}H]E_2 in the presence or absence of 1 μM unlabelled E_2 for ER or with 1 nM [^{3}H]1,25-$(OH)_2D_3$ in the presence or absence of 100 nM 1,25-$(OH)_2D_3$ for the 1,25-$(OH)_2D_3$ receptor. Apparent equilibrium was obtained under these conditions after 1 h for ER and 6 h for the 1,25-$(OH)_2D_3$ receptor [17, 18]. Binding was terminated by placing the trays on ice, aspirating the supernatant, and washing the monolayer for 20 min with ice-cold phosphate buffered saline containing 5% BSA. Cells were solubilized in 0.5 M NaOH–0.1% Triton X-100, and an aliquot was taken for estimation of radioactivity.

Statistical analysis

Paired Student's t-test was used to determine differences between treatment groups. The data presented here are expressed as means ± SEM.

RESULTS

The sensitivity of a series of 6 human breast cancer cell lines (4 ER + lines; MCF-7, MCF-7M, T-47D and ZR-75-1 and 2 ER − lines; MDA-MB-231 and BT-20) to the growth-inhibitory effects of RA was assessed by exposing cells to various concentrations of RA for a period equivalent to 4 population doublings of the untreated cultures. The data are presented in Fig. 1 and show that all cell lines, with the exception of MDA-MB-231, were growth inhibited by RA. Growth inhibition was in the order: T-47D > MCF-7M > ZR-75≏MCF-7≏BT-20 > MDA-MB-231. Growth inhibition was not apparent at concentrations of RA below 10 nM. In the two most sensitive lines (T-47D and MCF-7M) 50% growth inhibition was apparent at approx. 200–500 nM and continued to increase up to the maximal concentration tested, 10 μM.

Next, we examined interactions between RA and other growth inhibitory agents using T-47D cells. When these cells were exposed to various concentrations of RA and 10 nM 1,25-$(OH)_2D_3$, apparently synergistic effects were observed [Fig. 2(A)]. For instance, 10 nM 1,25-$(OH)_2D_3$ did not inhibit cell growth of T-47D cells significantly under these conditions, while 10 nM RA inhibited cell growth slightly i.e. by approx. 5–10%. However, simultaneous treatment with 10 nM RA and 1,25-$(OH)_2D_3$ inhibited cell growth by 20–30%. A similar phenomenon was observed when T-47D cells were exposed to

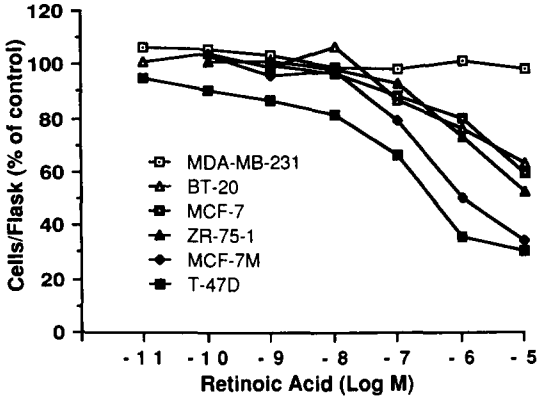

Fig. 1. Effect of various concentrations of RA on the proliferation of six human breast cancer cell lines. Cells (5×10^4) were plated into flasks in culture medium supplemented with 5% FCS. When cell numbers had doubled to approx. 10^5 cells/flask they were treated with vehicle alone or various concentrations of RA for a period equivalent to 4 population doublings of the untreated cultures. Triplicate flasks were then harvested and cell numbers were recorded. The cell number in RA treated cultures are expressed as a percentage of those in control cultures.

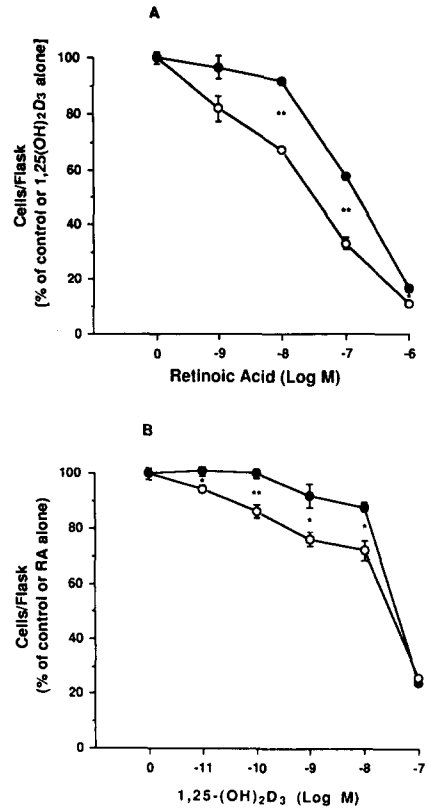

Fig. 2. Effects of combinations of RA and 1,25-$(OH)_2D_3$ on T-47D cell proliferation. (A) Effect of 1,25-$(OH)_2D_3$ on the sensitivity of T-47D cells to RA. Cells (5×10^4) were plated into flasks as in Fig. 1 and 24 h later treated with various concentrations of RA in the presence (open symbols) or absence (closed symbols) of 10 nM 1,25-$(OH)_2D_3$ for 6 days. $^*P < 0.01$, $^{**}P < 0.001$ RA alone vs RA + 1,25-$(OH)_2D_3$. (B) Effect of RA on the sensitivity of T-47D cells to 1,25-$(OH)_2D_3$. Cells (5×10^4) were plated into flasks and 24 h later treated with various concentrations of 1,25-$(OH)_2D_3$ in the presence (open symbols) or absence (closed symbols) of 10 nM RA for 6 days. Triplicate flasks were then harvested and cell numbers were recorded. Data are mean ± SEM of triplicate flasks. $^*P < 0.05$, $^{**}P < 0.01$ 1,25-$(OH)_2D_3$ vs 1,25-$(OH)_2D_3$ + RA. Where error bars are not shown, they did not exceed the size of the symbol.

various concentrations of 1,25-$(OH)_2D_3$ and 10 nM RA [Fig. 2(B)]. Treatment with < 10 nM 1,25-$(OH)_2D_3$ affected cell growth only slightly (< 5% of control) whereas in the presence of 10 nM RA concentrations of 1,25-$(OH)_2D_3$ > 0.1 nM inhibited cell growth significantly. When the above data were calculated following the formula outlined in Materials and Methods, the growth-inhibitory effect between RA and 1,25-$(OH)_2D_3$ was concluded to be synergistic. Since RA has been reported to increase 1,25-$(OH)_2D_3$ receptor concentrations in rat osteosarcoma cells [20], we examined the effect of RA on the 1,25-$(OH)_2D_3$ receptor levels in T-47D cells. RA pretreatment for 24 h did not increase specific 1,25-$(OH)_2D_3$ binding. At the highest

Table 1. Effect of various concentrations of RA on the specific [^{3}H]1,25-$(OH)_2D_3$ binding and the specific [^{3}H]E_2 binding in T-47D cell monolayers

Concentration of RA (M)	Specific [^{3}H]1,25-$(OH)_2D_3$ binding	Specific [^{3}H]E_2 binding
0	100 ± 1.4	100 ± 5.5
10^{-10}	98.3 ± 3.3	99.9 ± 6.9
10^{-9}	98.7 ± 1.6	107 ± 3.7
10^{-8}	99.4 ± 0.9	98.5 ± 3.1
10^{-7}	93.6 ± 5.4	100 ± 3.8
10^{-6}	80.8 ± 4.2*	70.7 ± 5.6*

Specifically bound [^{3}H]1,25-$(OH)_2D_3$ and [^{3}H]E_2 were determined as described in 'Materials and Methods' in cultures exposed to various concentrations of RA for 24 h. Specifically bound [^{3}H]1,25-$(OH)_2D_3$ or [^{3}H]E_2 is expressed as a percentage of that in control cultures. Data are mean ± SEM of triplicate wells.
*$P < 0.05$ vs control cultures.

concentration tested (1 μM) RA slightly but significantly decreased 1,25-$(OH)_2D_3$ binding (Table 1).

Interactions between RA and antioestrogens/oestrogens were also investigated. OHTam, a potent antioestrogen, inhibited growth of T-47D cells in a dose-dependent manner. The simultaneous exposure of 10 nM RA and various concentrations of OHTam resulted in apparently synergistic inhibition (Fig. 3). When the cell number was expressed as percent of control cells or cells treated with RA alone, the inhibition curve was shifted to the left by the treatment with RA, indicating a significant increase in sensitivity to OHTam in the presence of RA (Fig. 3 inset). When the data of RA and OHTam were calculated following the formula outlined in Materials and Methods, OHTam (10^{-10}–10^{-6} M) inhibited cell growth synergistically with 10^{-8} M RA. This phenomenon was

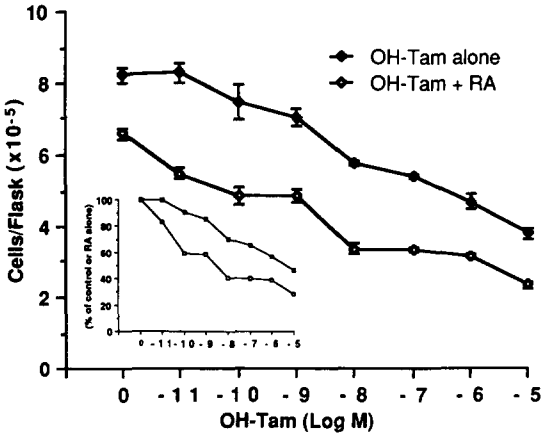

Fig. 3. Effect of RA on growth inhibition of T-47D cells by OHTam. Cells (5×10^4) were plated into flasks as in Fig. 1 and 24 h later treated with various concentrations of OHTam in the presence (open symbols) or absence (closed symbols) of 10 nM RA for 6 days. Triplicate flasks were then harvested and cell numbers were recorded. Data are mean ± SEM of triplicate flasks. Inset: Data are expressed as a percentage of those in control cultures (open symbols) or those in RA-treated alone cultures (closed symbols).

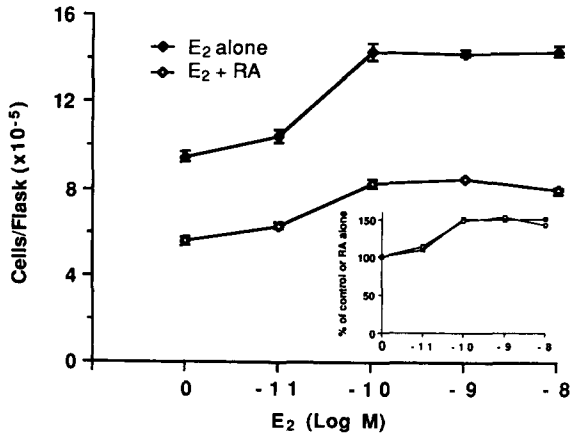

Fig. 4. Effect of RA on growth stimulation of T-47D cells by E_2. Cells (5×10^4) were plated into flasks containing 5 ml of phenol red-free medium supplemented with 5% dextran coated charcoal treated FCS instead of 5% FCS. Twenty-four hours later cells were treated with various concentrations of E_2 in the presence (open symbols) or absence (closed symbols) of 10 nM RA for 6 days. Triplicate flasks were then harvested and cell numbers were recorded. Data are mean ± SEM of triplicate flasks. Inset: Data are expressed as a percentage of those in control cultures (open symbols) or those in RA-treated alone cultures (closed symbols).

also observed when cells were treated with RA and other antioestrogens including OHClom and LY117018 (data not shown).

T-47D cell growth was stimulated by E_2 when these cells were cultured in phenol red-free RPMI 1640 containing 5% dextran coated charcoal treated FCS as shown in Fig. 4. When cells were treated simultaneously with 10 nM RA and various concentrations of E_2, RA appeared to have no effect on oestrogen sensitivity (Fig. 4). To examine the effect of RA on specific [^{3}H]E_2 binding, T-47D cells were pretreated with various concentrations of RA for 24 h. [^{3}H]E_2 binding was unaffected by treatment with 0.1–100 nM RA, but at 1 μM RA caused a significant decrease in [^{3}H]E_2 binding (Table 1).

DISCUSSION

Evidence has been accumulating which indicates that RA has a profound effect on the growth of human breast cancer cell lines in culture [7, 8]. The results in this study agree with previous work, which indicated that RA exhibits an antiproliferative effect at concentrations of 0.1–10 μM except in MDA-MB-231 cells. The mechanisms by which RA inhibits cell proliferation remain unclear. Recently Fontana *et al.* [21, 22] reported that RA inhibition of human breast cancer cell proliferation is accompanied by inhibition of synthesis of a

39K protein and stimulation of the synthesis of 75 and 46K proteins. However, the potential role of these proteins as autocrine growth factor(s) or growth-inhibitory factor(s) remains to be clarified.

Analysis of cDNA clones for retinoic acid receptors (RARs) indicate that RARs are members of the steroid/thyroid receptor gene family [23, 24]. Recently, three forms have been characterized in the human (hRAR-α, -β and -γ) [23, 25, 26]. T-47D cells have been reported to contain mRNA transcripts for the -α and -γ forms [26]. This suggests that RA modulates the action of steroid hormones in human breast cancer cells via RAR-mediated mechanisms.

Our studies revealed that RA acts synergistically with 1,25-$(OH)_2D_3$ or a series of antioestrogens to inhibit T-47D breast cancer cell proliferation. In rat osteosarcoma cells, RA increased 1,25-$(OH)_2D_3$ receptor [20], but RA did not enhance the action of 1,25-$(OH)_2D_3$ [27]. In contrast, RA enhanced the growth-inhibitory effect of 1,25-$(OH)_2D_3$ in T-47D cells without a change of 1,25-$(OH)_2D_3$ receptor levels. These results indicate synergism occurs at a level beyond the modulation of receptor concentrations. Recently synergistic action between glucocorticoid hormone and E_2 at the level of hormone responsive elements in DNA has been reported [28]. RA has been shown to act synergistically with glucocorticoid or thyroid hormone to stimulate synthesis of growth hormone [29] and Bedo *et al.* [30] showed that these synergistic actions also occurred when the growth hormone gene promoter was linked to the chloramphenicol acyltransferase reporter gene. It is interesting to speculate that synergism between RA and 1,25-$(OH)_2D_3$ may occur at the level of putative hormone responsive elements within autocrine growth factor genes in breast cancer cells. However, at this time it is unclear whether or not steroidal regulation of human breast cancer cell proliferation is mediated entirely through autocrine growth factor production. Further detailed studies are required to clarify the molecular basis of growth modulation by RA and 1,25-$(OH)_2D_3$.

The other interesting observation reported in this communication is that RA also acts synergistically with the inhibitory effect of antioestrogens but not with stimulatory effects of E_2. However, in other studies RA and OHTam produced only an apparently additive effect on growth inhibition of T-47D cells *in vitro* [31, 32]. The reason for this apparent discrepancy is not clear. The mechanism of synergistic action between RA and antioestrogen is not known but is unlikely to involve direct effects of RA on ER. First, there was no synergistic action between RA and E_2 and second, RA had effects on ER levels only at 10^{-6} M while synergistic inhibition was apparent at 10^{-8} M. More recent data raise the possibility that the observed synergism between antioestrogens and RA is mediated via effects on autocrine growth factor production. A study with MCF-7 cells demonstrated that transforming growth factor-β (TGF-β) could potentiate the inhibitory effects of RA on cell proliferation [33]. Since it has previously been shown that antioestrogens increase the secretion of active TGF-β by MCF-7 cells [34], a case can be made for TGF-β acting as an important intermediary in the synergism between antioestrogens and RA. Further detailed studies are required to clarify this potential mechanism and to determine the cellular specificity of these effects.

In summary, in T-47D human breast cancer cells RA inhibits cell proliferation synergistically with 1,25-$(OH)_2D_3$ and antioestrogen. These results suggest that clinical trials utilizing combination therapy of RA with antioestrogens and possibly vitamin D_3 analogues may provide a more beneficial response in some hormone-responsive breast tumours.

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