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The data presented support our hypothesis that retinoids inhibit human breast cancer cell proliferation by inducing a state of functional mitogen deprivation. Kinetic and quantitative changes in cell cycle progression and gene expression following retinoic acid treatment of the estrogen dependent, T-47D breast cancer cell line, are consistent with retinoic acid inducing a block in the treated cells' ability to respond to external mitogenic signals, specifically to epidermal growth factor. The data further implicate protein kinase Cα as a retinoid induced gene product necessary for inhibiting EGF signaling. By manipulating the expression of the cellular retinoic acid binding protein, type II, the cellular retinol binding protein and the nuclear retinoic acid receptor alpha, we also have shown that expression of these mediators of vitamin A action is sufficient to exert growth inhibitory effects on human breast cancer cells in culture.

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Introduction.

The project funded as DAMD17-94-J-4100, had two purposes. First, it was to provide support, in the form of a pre-doctoral fellowship, for training Ms. Ann P. Tighe as an active member of the breast cancer research community. This has been accomplished. Ms. Tighe is within six months of defending her doctoral dissertation. In addition, she is second author on one publication, and as first author is preparing two manuscripts for submission. She has also presented her results, in poster format, at the 1996 FASEB summer research conference on Retinoids, the 1997 Conference of Retinoids sponsored by the European Retinoid Research Group in Nice, France (9/28/97 - 10/1/97), and will present a poster at the 1997 Era of Hope meeting sponsored by the Department of Army's Breast Cancer Research Program (Nov. 2, 1997) in Washington DC.

Second, we proposed as part of Ms. Tighe's thesis research, to accomplish four Technical Objectives: 1. To define in detail, the expression of cellular retinoid-binding proteins and nuclear retinoid receptors in human mammary carcinoma cell lines; (2) To determine the degree of responsiveness of these cell lines to retinoic acid and retinol; (3) To determine if the enhanced retinoid responsiveness of interferon treated mammary carcinoma cells results from altered expression of cellular retinoid-binding proteins or nuclear retinoid receptors; and (4) To determine if altered expression (increased or decreased) of cellular retinoid-binding proteins or nuclear retinoid receptors alters the biological responsiveness of human mammary carcinoma cells to retinoic acid or to retinol. As documented below, these objectives have largely been met, and further, these studies have provided details of the mechanism by which retinoic acid inhibits the proliferation of at least one, human breast cancer cell line.

Background

The anti-tumorigenic action of retinoids can be accounted for both by inhibition of cell proliferation and induction of cell differentiation (Lotan, 1996). Links between dietary vitamin A status and breast cancer development have resulted in clinical trials to evaluate the efficacy of retinoids in the prevention and therapy of breast cancer (Costa, 1993). The ability of retinoic acid (RA) to affect a target cell depends on the action of cytoplasmic retinoid-binding proteins and nuclear, ligand-dependent transcription factors (primarily the RARs) (Gudas et al., 1994; Mangelsdorf et al., 1994). Expression and activity of these retinoid binding proteins and nuclear receptors may be critical in regulating the biological response and tissue sensitivity to retinoids at the cellular and molecular level (Gudas et al., 1994). Understanding the cellular and molecular mechanism of retinoid action could provide a basis for more effective retinoid therapy of breast cancer and a greater understanding of the role of retinoids in the etiology of breast tumor development.

Retinoids inhibit the proliferation of hormone-dependent, but not hormone-independent human breast carcinoma (HBC) cell lines (Liu et al., 1996; Wilcken et al., 1996). Proliferation of normal and malignant mammary epithelial cells is regulated by the interplay of hormonal and growth factor signaling pathways that regulate cell cycle progression (Dickson and Lippman, 1995). Two HBC cell lines are used in our study. The steroid hormone-dependent T-47D cell line, does not grow in the presence of retinoids, whereas growth of the hormone-independent MDA-MB-231 line is not affected by retinoids. The experiments in this project were designed to determine the molecular basis for the different biological responses of these two HBC cells to retinoids.

Methods

Analysis of retinoid binding protein and retinoic acid receptor expression. The expression of CRABP I, CRABP II, CRBP I, and RARs α, β and γ were compared in 5 human breast cell lines, 4 derived from breast tumors (T47-D, MCF-7, BT20 and MDA-MB-231) and

one an immortalized, "normal" cell line (MCF10A). Cells were grown in standard tissue culture media as recommended by the American Type Culture Collection. Total RNA was isolated and expression monitored by northern blot analysis (Talmage and Lackey, 1992; Cho et al., 1997). Results of this analysis are summarized in Table 1.

Determination of Cell Cycle Distribution. Effects of retinoids on cell proliferation were monitored by following changes in total cell number, cell viability, and by determining the number of cells containing 2N, 4N or >2N, <4N DNA contents. Cell number and viability was measured following trypsinization of cells and staining with trypan blue. The number of cells staining with trypan blue (% non-viable) or excluding trypan blue (% viable) were counted with a hemocytometer. DNA content was measured after staining fixed cells with propidium iodide, and analysis with a fluorescence activated cell sorter. Data was analyzed using a ModFit software package.

Analysis of Immediate Early Gene Induction. The induction of the c-fos, c-jun and c-myc immediate early response genes, and the delayed response gene, cyclin D1 was followed by northern blot analysis, or by immunoblotting as previously described (Talmage and Lackey, 1992).

Analysis of Protein Kinase Activity. Activities of the p70 and p90 ribosomal S6 kinases, the mitogen activated protein kinases, Erk1 and Erk2, and the stress activated protein kinases, JNK and p38, were measured in immune complex kinase reactions (antibodies were obtained from Upstate Biotechnology Inc., and from Santa Cruz Biotech., Inc.). Cell extracts containing 250 μg total protein were immunoprecipitated with 1 μg kinase specific antibody. Immune complexes were collected with protein A-agarose beads, washed extensively, and then incubated in the presence of MgATP (γ-^{32}P) and peptide substrates. Transfer of ^{32}P to substrates was measured by scintillation counting following spotting of reaction mixtures onto phosphocellulose paper squares, and washing in 1% phosphoric acid.

Analysis of Epidermal Growth Factor Receptor Function. Activation of the EGF receptor following EGF treatment of cells was measured in either immunecomplex kinase reactions, or by determining the phosphotyrosine content of immunoprecipitated EGF receptor.

Results

Task 1. To define in detail, the expression of cellular retinoid-binding proteins and nuclear retinoid receptors in human mammary carcinoma cell lines. Previously, we used northern blot analysis to compare the expression cellular retinol- and retinoic acid binding proteins and nuclear retinoid receptors between T-47D (RA-sensitive) and MDA-MB-231 (RA-insensitive) cells (Table 1). As a result of these studies, our work focused on the role of RARα, CRABP-II and CRBP in mediating the antiproliferative effects of retinoids because: (a) RARα and CRABP-II expression vary most between RA-sensitive and RA-resistant cells; (b) in vivo RARα expression is inversely related to the severity of neoplastic breast disease; (c) we have established that RARα can impart RA sensitivity on RA-resistant cells and inhibits transformation by blocking immediate early gene expression (Talmage and Lackey, 1992; Talmage and Listerud, 1994); and (d) CRBP expression varies during hormonally regulated mammary development (Morrison and Leder, 1994) and we have demonstrated that similar regulation alters retinol (ROL) responsiveness in the cervix (Tannous-Khuri et al., 1994; Tannous-Khuri and Talmage, 1997).

Table 1. Summary of the expression of retinoid binding proteins and receptors in mammary cells in vitro and in vivo.

	RA-SENSITIVE (T47-D)	RA-RESISTANT (MDA-MB-231)	MAMMARY GLAND
RARα	+++	+/-	++
RARβ	-	-	+
RARγ	+	++	-
CRBP I	-	-	+
CRABP I	-	-	ND
CRABP II	+++	-	ND
ER	+	-	+/-

Expression of the indicated genes was determined by northern blot analysis of total RNA (cell lines), or by in situ hybridization (mammary gland).

Task 2. To determine the degree of responsiveness of these cell lines to retinoic acid and retinol. Treatment of T-47D cells with nanomolar RA resulted in proliferation arrest, whereas micromolar concentrations of RA had no effect on MDA-MB-231 cell proliferation (Figure 1).

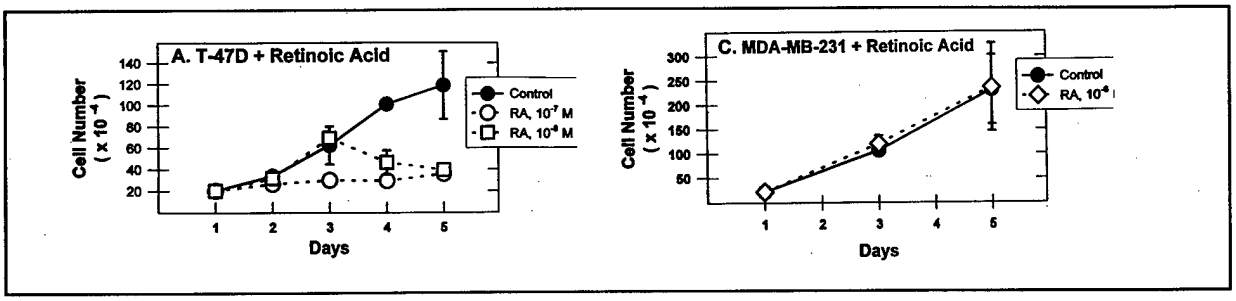


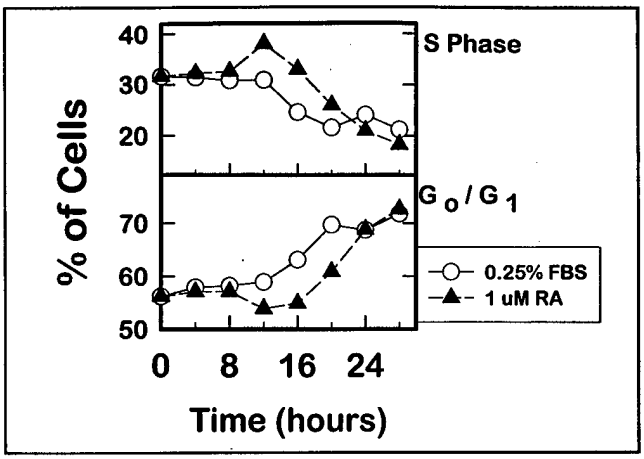
Figure 1. Hormone dependent (T47-D, Panel A) or hormone independent (MDA-MB-231, Panel B) human breast cancer cells were plated on day 0. Twenty-four hours later vehicle (0.01% ethanol) or the indicated concentrations of retinoic acid were added. Cell numbers were determined on the indicated days.

As a result of the differential sensitivity of these cell lines to the anti-proliferative effect of RA, we used various measures of cell cycle progression in response to retinoids, to compare the activity of RA, and retinol (ROL) in sensitive and insensitive cell lines.

- **RA arrested hormone-dependent T47-D cell proliferation prior to the G1/S checkpoint in the cell cycle** (Fig. 2).

Retinoids arrest T-47D cell proliferation. Based on flow cytometry of propidium iodide stained cells, we concluded that RA-induced growth arrest of T-47D cells was associated with an accumulation of cells in the Go/G1 phase of the cell cycle. Arrest in Go/G1 typically is associated with either a block to transit through the restriction point in late G1, or to withdrawal of cells from the cell cycle. Withdrawal can reflect either quiescence, terminal differentiation or programmed cell death. The observation that RA-induced growth arrest was fully reversible, coupled with the lack of apparent fragmented nuclear DNA in the flow cytometry profiles, rules out either terminal differentiation or apoptotic cell death. To determine if RA-induced arrest occurred at the G1/S restriction point, or resulted from induction of quiescence, we measured the effect of RA on the expression of genes involved in progression through early G1 (the immediate early response genes, c-fos, c-jun and c-myc) or through mid-to-late G1 (cyclin D1). In addition we compared the temporal relationship between RA alteration of the expression of these genes, with that of mitogen deprivation (following serum withdrawal).

Figure 2. T47-D cells growing in the presence of 10% FBS were treated at T=0 with RA (0.1 μM; filled symbols), or they were serum depleted (0.25% FBS, open symbols). At 4 h intervals the distribution of cells in each cell cycle stage were quantified by propidium iodide staining and FACS analysis. The data are shown as the change in the total percent of each cell population in S phase (top) or Go/G1 (bottom) relative to untreated controls. Serum deprivation (after 12 h) and retinoic acid treatment (after 16 h) prevented cell entry into S, and caused cells to exit the cell cycle in Go/G1.



- **RA attenuated all measured responses to EGF including transcriptional activation of c-fos, c-jun and c-myc; and activation of signaling protein kinases (Fig. 3 & 4).** Effects of RA were apparent within 3-6 h (Fig. 3 C) and were blocked by inhibiting protein synthesis with cycloheximide (not shown). RA did not affect EGF or FBS signaling in MDA-MB-231 cells.

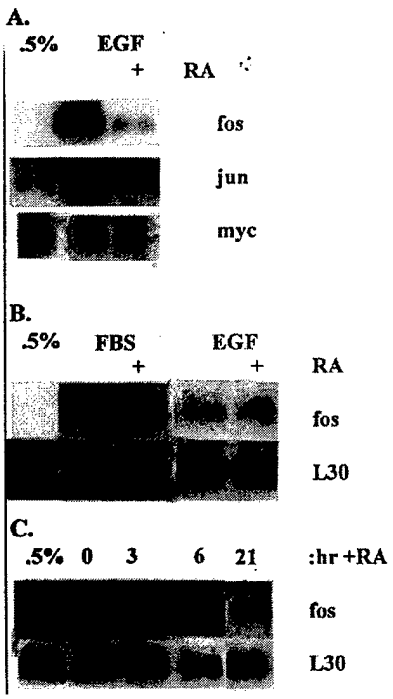


Figure 3. T47-D cells (A) or MDA-MB-231 cells (B) were deprived of FBS and EGF for 24 h, in the presence or absence of 10^{-7} M RA (RA+). EGF (10 ng/ml; EGF+) or FBS (10%; FBS+) were added for 20 min. c-fos, c-jun and/or c-myc gene induction was determined by northern blot analysis. Blots were probed with the L30 ribosomal protein cDNA as a control. In C, RA was added to serum deprived T47-D cells for the indicated times prior to stimulation with EGF.

The relevance of these molecular measures of RA action was supported by the observation that in MDA-MB-231 cells which are resistant to the anti-proliferative action of retinoids, RA treatment had no effect on the level of mitogen-induced immediate early gene expression (Figure 3B).

Accumulation of cells in Go/G1 phase and a concomitant decrease in DNA synthesis was first evident between 16 to 20 hours after RA addition, compared to equivalent changes seen 8 hours after serum withdrawal (Figure 2). The 8 hour delay in the RA response compared

to mitogen withdrawal is consistent with the need for sufficient expression of RA-induced gene products that inhibit cell cycle progression. The decrease in the percent of treated cells in S phase was preceded by a fall in the expression of cyclin D1. Decreased D1 (mRNA and protein) was apparent at 6 and 9 hours after serum withdrawal or RA addition, respectively (not shown). In contrast, to these delayed responses, when we measured mitogen induction of c-fos, c-jun and c-myc expression by northern blot analyses, we found that a 3-6 hour treatment with RA repressed immediate early gene (IEG) induction (Figure 3C). This effect of RA required new protein synthesis since it was prevented by co-treatment with cyclohexamide.

EGF binding to the epidermal growth factor receptor (EGFR) induces receptor dimerization with other erbB receptors such as erbB2 and erbB3. Following ligand-dependent activation, receptor tyrosine kinases such as the EGF receptor, autophosphorylate and the resulting cytoplasmic phosphotyrosines bind to and activate signaling molecules such as the Ras GTP-binding protein and phosphatidylinositol 3-kinase (PtdIns 3-kinase). Activation of each of these pathways leads to activation of cytoplasmic and nuclear serine/threonine protein kinases which alter the expression of genes intimately involved in cell cycle regulation. To investigate further which growth factor activated signaling pathways are sensitive to RA, we assayed protein kinases that are known to be activated by mitogenic stimuli. Retinoic acid pre-treatment inhibited EGF induction of MAPK, p70 S6 kinase, Jun N-terminal kinases (JNK) and p38 kinase (Figure 4).

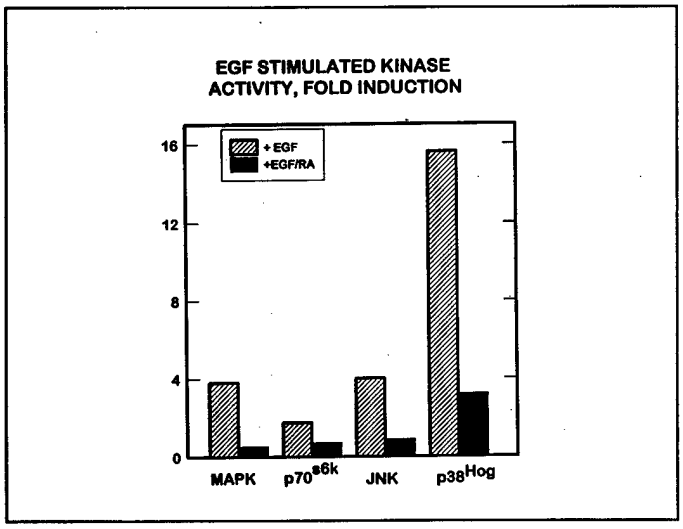


Figure 4. Serum starved T-47D cells were stimulated with 10 ng/ml EGF for 30 min. following a 24 hr pre-treatment with 10^{-6} M RA, or ethanol control (C). Lysates (200μg protein) were immunoprecipitated with specific antibodies recognizing the indicated kinases. After washing, immune complexes were incubated with ^{32}P-γ–ATP and peptide substrates. Kinase activity was quantified by scintillation counting. The data are presented as the fold stimulation over activities in serum starved cells.

RA and the phosphatidylinositol 3-kinase (PtdIns 3-kinase) inhibitor wortmannin had similar effects on T47-D cell proliferation (Figure 5). This observation is consistent with other studies demonstrating that RA inhibits de novo transformation of cells by disrupting a signal transduction pathway leading from PtdIns 3-kinase to the c-fos promoter (Chen et al., 1997; Talmage and Lackey, 1992; Talmage and Listerud, 1994).

JNK, p38 and p70^{s6k} can be activated following receptor tyrosine kinase activation of PtdIns 3-kinase. RA inhibits transformation of rat fibroblasts by preventing oncogene signaling to the c-fos promoter (Talmage and Listerud, 1994). In these cells, RA inhibits c-fos transcriptional activation by preventing PtdIns 3-kinase-dependent activation of JNK (Chen et al., submitted). If RA inhibits growth of T-47D cells in a similar fashion, then inhibition of this pathway by other agents should also inhibit proliferation. Therefore, we compared the antiproliferative effects of wortmannin, a PtdIns 3-kinase inhibitor, and rapamycin an immunosupressive compound that can inhibit proliferation by preventing activation of p70^{s6k} with the effect of RA on proliferation, cell cycle progression and IEG induction (Figure 5).

All three agents inhibit cell proliferation (Figure 5a) and arrest cells in G_0/G1 (Figure 5b). Wortmannin and RA, but not rapamycin, also prevent mitogen activation of IEG expression (Figure 5c). Therefore, repression of EGF-induced c-fos and c-jun gene expression distinguishes the cell cycle arrest and antiproliferative effect of RA and wortmannin from rapamycin. Since MDA-MB-231 cells are resistant to RA, we determined if these cells were also resistant to wortmannin-induced inhibition of proliferation. Wortmannin inhibited neither proliferation nor mitogen induction of IEGs in MDA-MB-231 cells (data not shown).

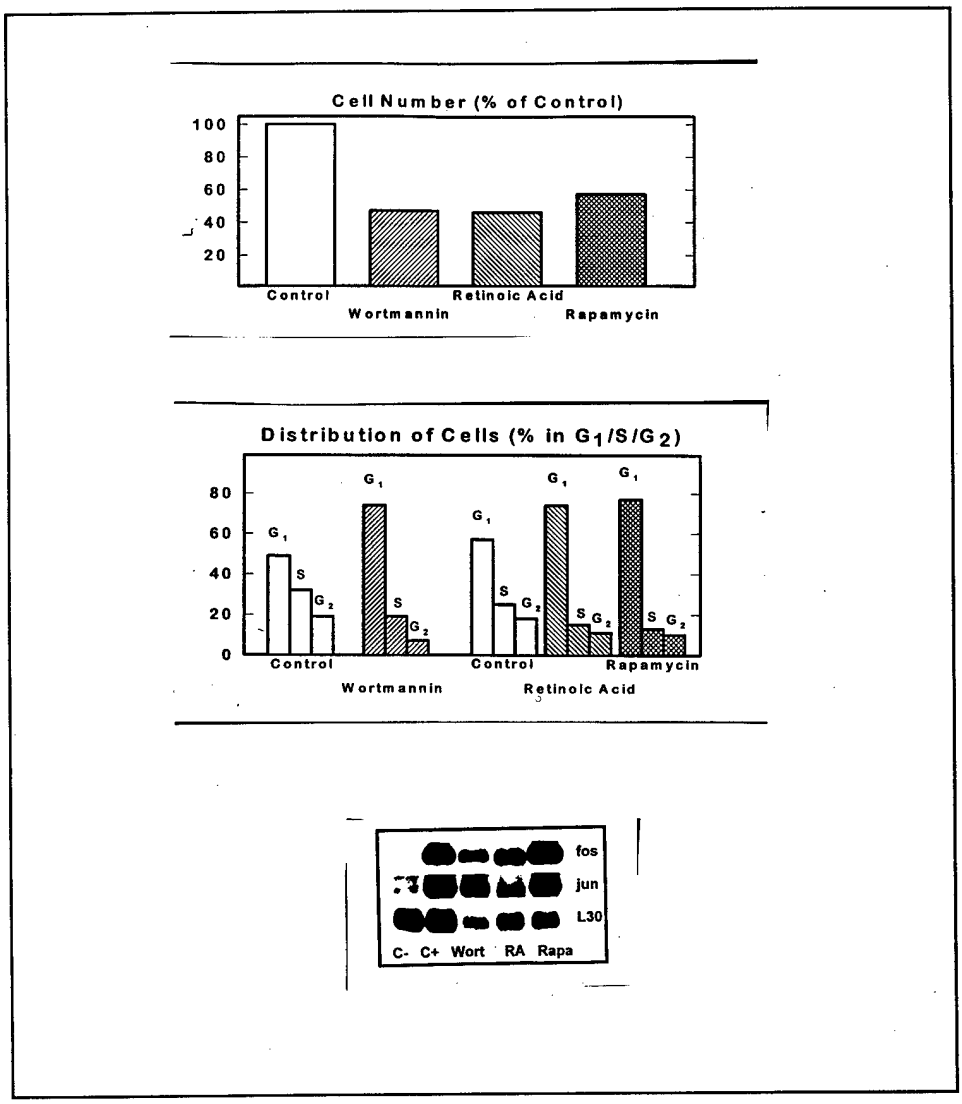


Figure 5. a. A total of 10.5 x 10^4 cells were seeded in 60 mm dishes and treated with 10^{-6} M wortmannin, 10^{-7} M RA or 20 ng/ml rapamycin for 5 days. Media and experimental treatments were renewed every 72 hours. On day 5, cells were trypsinized and counted with a hemocytometer. Cell viability was determined by trypan blue exclusion (90+% at all times). Error bars show standard deviations.

b. Serum starved T-47D cells were stimulated to re-enter the cell cycle with 10 ng/ml EGF and 10% FBS, with or without 10^{-6} M wortmannin (left). Asynchronously growing T-47D cells were cultured in 5% FBS with or without 10^{-6} M RA or 20 ng/ml rapamycin (right). For the release experiment, cell cycle phase distribution was determined by FACS analysis after 24 hr. For RA or rapamycin treated cells, FACS analysis was performed after 44 hr.

c. Northern blot analysis of total RNA (15 mg each) isolated from serum-starved T-47D cells 30 min. after stimulation with 10 ng/ml EGF. Cells were pretreated with 10^{-6} M RA, 10^{-6} M

wortmannin or 20 ng/ml rapamycin for 16 hr prior to EGF stimulation. Filters were probed with ^{32}P-cRNAs as described in the legend to Figure 3.

We interpret these data as follows: RA-induces the expression of a gene or genes whose product inhibits cell cycle progression. The RA-induced gene(s) inhibits the EGF receptor signaling preventing induction of the AP-1 (Fos/Jun) and/or Myc transcription factors that are required for expression of the G1 cyclins. Failure to express cyclin D1 prevents cells from progressing through G1 and results in cell cycle arrest. The identity of the genes that are regulated by RA in these cells continues as an area of active research. One candidate, the gene encoding the alpha isotype of protein kinase C (PKCα), has been identified and investigated.

In a recent publication (Cho et al., 1997), we demonstrated that:

- **RA induced expression of PKCα in T47-D cells (no other conventional PKCs are expressed in T47-D cells, either in the presence or absence of RA)**.
- **RA-independent expression of PKCα significantly reduced proliferation of T47-D cells**.
- **Inhibition of PKCα activity reversed the anti-proliferative effects of retinoic acid.**

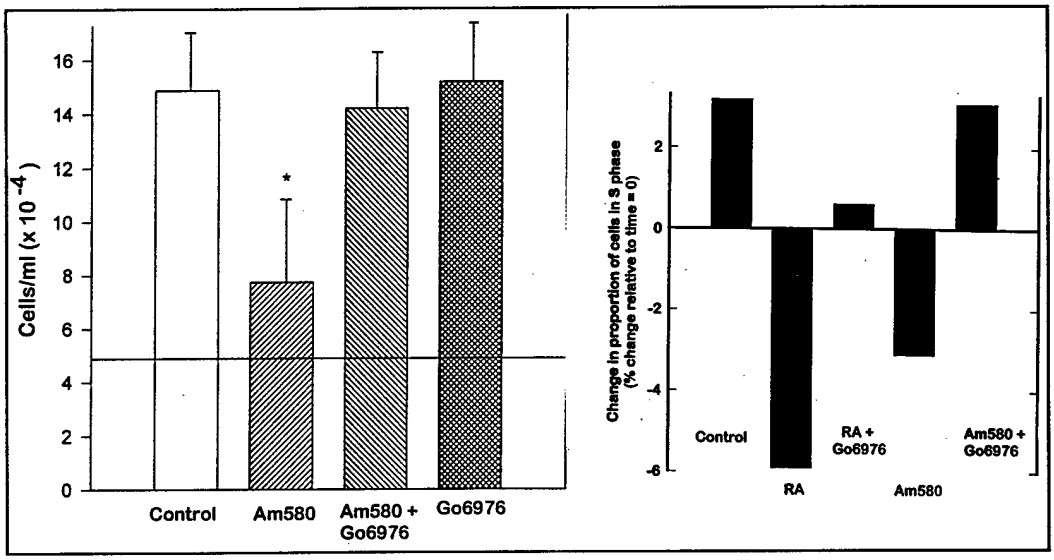


Figure 6. T47-D cells were treated with RA, the RARα selective synthetic retinoid, Am580, the conventional PKC inhibitor, Go6976, or combinations of retinoid and inhibitor for 3 days, after which cell numbers were measured (A) and cell cycle phase distribution (by FACS analysis, % of cells in S phase is shown, B) was measured. The conventional inhibitor of PKCs partially, or completely reversed the negative proliferative effects of retinoids in these cells.

More recently we (Tighe and Talmage, in prep) have found that:

- **RA prevents EGF activation of the EGF receptor** (Fig. 7).
- **Inhibition of PKCα activity restored EGF signaling in RA-treated cells** (Fig. 7).

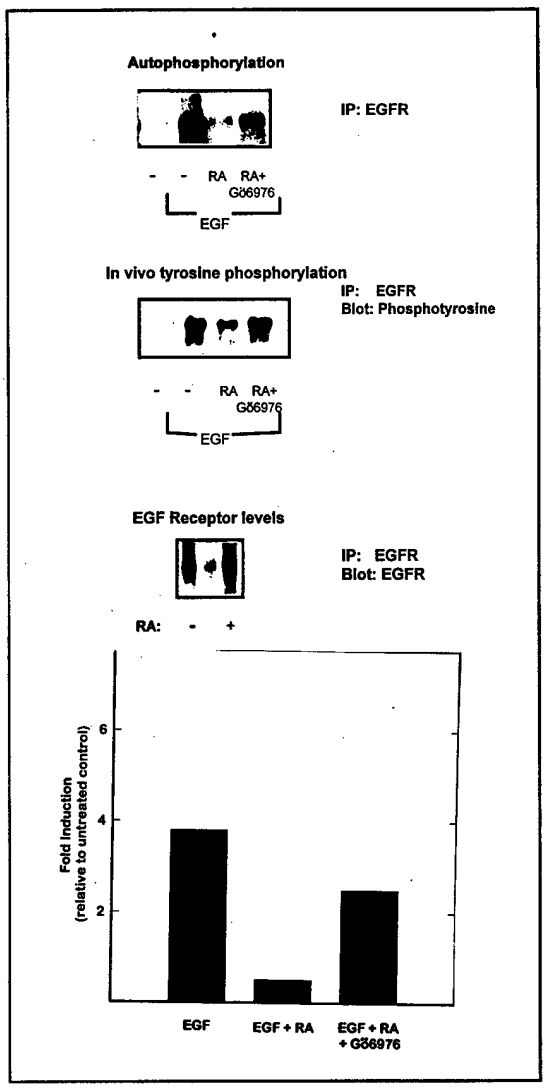


Figure 7. T47-D cells were serum and EGF deprived for 24 h in the presence or absence of 10^{-7} M retinoic acid (RA). Cells were stimulated for 10 min. with EGF (10 ng/ml). EGF receptor was immunoprecipitated from whole cell extracts and either incubated with ^{32}P-γ-ATP (Top), or resolved by SDS-PAGE, transferred to nitrocellulose and probed with either an antibody that recognizes phosphotyrosine (Middle) or that recognizes the EGF receptor (Bottom). Antibody-antigen complexes were visualized using ECL. Some cultures were treated with the inhibitor of conventional PKCs, Go6976 (250 nM), at the same time as RA. RA pretreatment reduced EGF activation of the EGFR tyrosine kinase and reduced in vivo tyrosine phosphorylation of the EGFR without affecting total EGFR levels. Inhibition of PKCα activity during the RA pre-treatment period reversed the effects of RA on EGF activation of the EGFR.

Based on these findings we propose the following model for the anti-proliferative action of RA on hormone-dependent, human breast cancer cells. RA induces expression of the PKCα isozyme (Cho et al., 1997; Khuri et al., 1996). PKCα phosphorylates the cytoplasmic domain of the EGF receptor reducing its affinity for EGF (phosphorylation of the EGF receptor on threonine residue 654 by PKC has been established as a means of attenuating EGF signaling in other cell types; Chen et al., 1996). Threonine-654 phosphorylated EGF receptors fail to transduce mitogenic signals via PtdIns 3-kinase resulting in exit from the cell cycle. RA does not inhibit hormone-independent MDA-MB-231 cell proliferation: (a) because RA does not induce PKCα expression in these cells, and (b) because EGF is not an important growth factor for MDA-MB-231 cells (Fig. 3B).

Task 3. To determine if the enhanced retinoid responsiveness of interferon treated mammary carcinoma cells results from altered expression of cellular retinoid-binding proteins or nuclear retinoid receptors. Cytokines, such as interferon-gamma (IFNγ) have attracted attention for their potential to synergize with retinoids as anti-tumor agents. We proposed to determine if the reported synergism between retinoids and γ–INF in hormone-independent breast cancer cells resulted from induction of CRBP I, CRABP II or RARα expression by interferon-induced transcription factors. We have demonstrated that:

- <u>γ-INF did not change the expression of CRBP-I, CRABP-II or RARα</u> in either cell line. and further, retinoic acid and γ–INF did not synergistically inhibit MDA-MB-231 cell proliferation (not shown).

Task 4. To determine if altered expression (increased or decreased) of cellular retinoid-binding proteins or nuclear retinoid receptors alters the biological responsiveness of human mammary carcinoma cells to retinoic acid or to retinol. We have demonstrated that the inhibition of IEG expression and cell cycle progression correlate with the growth-

inhibitory effect of both RA and ROL on T-47D cells (Figure 8). The antiproliferative effect of ROL is consistent with its role as a precursor for RA (Figure 8a). The lower sensitivity of T-47D cells to ROL compared to RA is consistent with the low uptake of ROL by these cells (data not shown). The magnitude of the effects of ROL of cell cycle changes (Figure 8b) and IEG expression (Figure 8c) parallels the degree of ROL-induced growth inhibition further supporting a mechanistic link between the anti-proliferative effects of retinoids and modulation of immediate early gene expression.

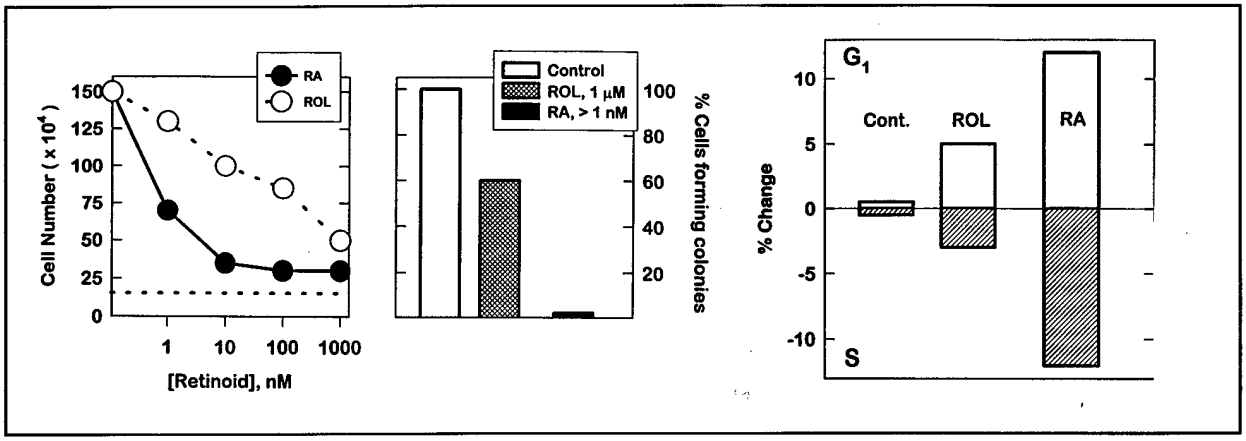


Figure 8. T47-D cells were cultured in the presence of the indicated concentrations of either retinoic acid (RA) or retinol (ROL). A. Cells initially were plated at 15 x 10^4 cells/plate (indicated by the dotted horizontal line). After 5 d cell numbers were measured. The results establish that both retinoic acid and retinol inhibit hormone-dependent breast cancer cell proliferation ($IC50_{RA}$ = ~ 1 nM; $IC50_{Rol}$ = ~100 nM). B. T47-D cells (1000) were plated on 60 mm dishes in the presence of retinoic acid (RA, 10^{-9} M), retinol (ROL, 10^{-6} M), or without retinoids (control). After 21 days colonies were counted. Data are shown as the percent of plated cells that gave rise to colonies. T47-D cells have a 100% cloning efficiency. Retinol reduced T47-D cloning efficiency by 40%. No colonies were observed in the presence of 1 nM retinoic acid, even when 10,000 cells were initially plated (not shown). C. Serum starved parental T-47D cells were stimulated to re-enter the cell cycle with 10 ng/ml EGF and 10% FBS at time 0. After 5 hr, 10^{-6} M RA or 10^{-6} M ROL was added and cell cycle phase distribution determined by FACS after an additional 24 hr.

We have proposed that CRBP is necessary for efficient retinol utilization by target cells and that the lack of CRBP expression in T-47D cells might account for the minor effect of ROL on these cells compared to RA. Therefore, we reasoned that transfecting T-47D cells with an expression vector encoding CRBP should increase the ability of retinol to inhibit proliferation. We isolated and have completed proliferation assays on T-47D-CRBP expressing cells.

Studies on these cell lines led to the following observations:

- **CRBP-I expression affected T47-D proliferation in a manner similar to** the effects of treating the parental T47-D cells with **10^{-6} M retinol or 10^{-9} - 10^{-8} M RA** (Figures 9 & 10).

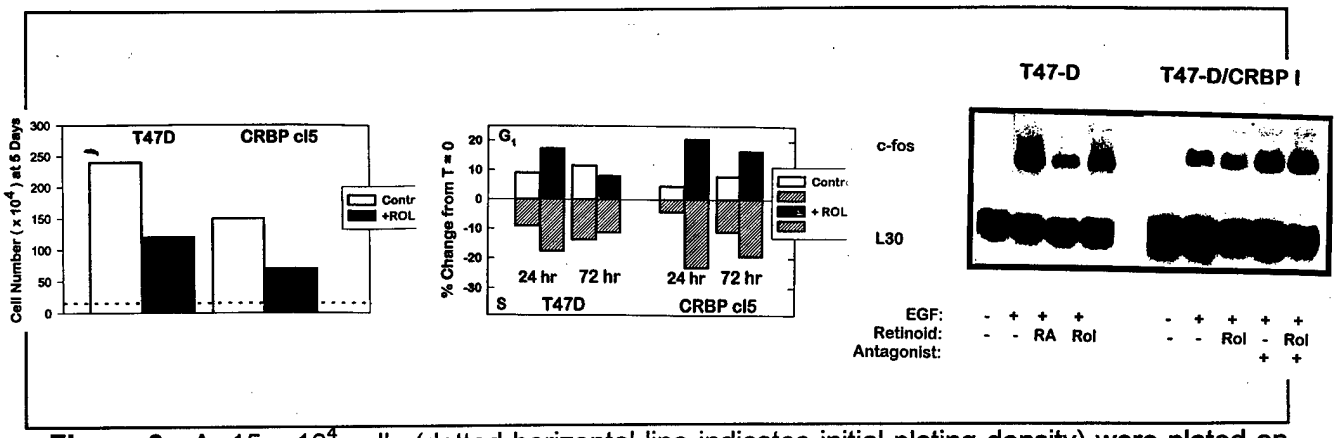


Figure 9. A. 15 x 10^4 cells (dotted horizontal line indicates initial plating density) were plated on 60 mm dishes and treated with 10^{-6} M ROL. Cell numbers and viability were measured after 5 days. Viability was 90+% at all times. Both CRBP I expression and retinol treatment increased the population doubling time of T47-D cells from ~30 hr. to ~40 hr. (i.e. by >30%).

B. Serum starved parental T-47D cells, or CRBP I expressing T47-D cells (clone 5) were stimulated to re-enter the cell cycle with 10 ng/ml EGF and 10% FBS at time 0. After 5 hr 10^{-6} M ROL was added to half the plates (shaded bars) and cell cycle phase distribution determined by FACS after 24 and 72 hr.

C. Parental T47-D (left) or CRBP-I transfected T47-D (right) cells were serum/EGF deprived for 24 h. As indicated, either retinoic acid (RA, 10^{-7} M), retinol (Rol, 10^{-6} M) or the RARα antagonist, Ro41-5153 (Antagonist +, 10^{-5} M; Keidel et al., 1994) were added during this time. Cells were then stimulated with EGF (10 ng/ml) for 20 min. and c-fos expression was measured by northern blotting.

EGF induced c-fos expression more in control parental cells than it did in control CRBP-I expressing cells. RA and retinol inhibited EGF-induction of c-fos in the parental cell line. Retinol had no further effect beyond that achieved by expressing CRBP-I in the T47-D cell line (T47-D/CRBP-I). Treatment of T47-D/CRBP-I cells with the RARα antagonist Ro41-5153, restored EGF induction of c-fos expression, in the presence and absence of 1 μM retinol.

Although the relative decrease in cell number in response to ROL treatment was not greater in T-47D-CRBP cells compared to parental T47-D cells, both the magnitude and duration of decreased progression into S phase of ROL-treated T-47D-CRBP cells was greater than in ROL-treated parental cells (Figure 9), and the total cell number of untreated T-47D-CRBP cells after 5 days is lower than in the untreated parental cells (Figure 9). This slower rate of growth and prolonged response to ROL, might reflect a CRBP-mediated change in the uptake and/or utilization of ROL from the media. This conclusion was supported by data indicating that EGF poorly induced fos expression (Figure 9C), and poorly activated JNK and p38 kinases (not shown) in T-47D-CRBP cells, and further, that treatment with the RARα antagonist restored EGF responsiveness to these cells.

Although γ- interferon failed to synergize with retinoic acid in MDA-MB-231 cells (Task 3, above), it did synergize with both low concentrations of RA and with retinol, in T47-D cells. In contrast to the reversible growth arrest achieved with retinoids alone (or with γ-interferon alone), combined treatment with γ-interferon and retinoid killed growth arrested cells (Figure 10). Surprisingly, CRBP I expression prevented the lethal effect of combined retinol and γ-interferon treatment (Fig. 10C).

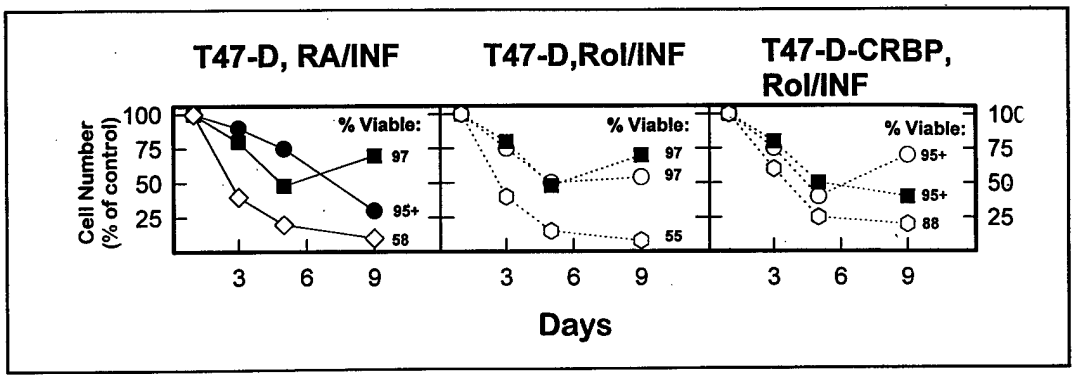


Figure 10. T47-D cells, or T47-D-CRBP I cells were plated in the presence of 10^{-9} M RA, 10^{-6} M retinol (ROL) or 100 U/ml of γ-interferon, or combinations of retinoid and interferon. Cell number and viability was measured on the days indicated. Cell numbers are plotted relative to vehicle treated cells at each time point. Until day 5 viability in all conditions was >90%. Viability on day 9 is shown.

Studies with the RARα selective agonist, Am580, indicate that this receptor isoform mediates the anti-proliferative effect of RA (Cho et al., 1997). This conclusion is supported by demonstrating that pretreating T-47D cells with Ro41-5153, an RARα selective antagonist, abrogated the ROL-induced repression of c-fos induction in T-47D-CRBP cells and causes a superinduction of c-fos in the parental cells. Since the RA-resistant MDA-MB-231 cell line expresses only low levels of RARα we transfected these cells with a vector encoding the human RARα cDNA. In addition, MDA-MB-231 do not take up RA from the culture media efficiently. We also found that these cells do not express CRABP II (Table 1). Since CRABPs might influence the uptake and metabolism of serum retinoids, we transfected both MDA-MB-231 and RARα expressing MDA-MB-231 cells with CRABP II expression vectors. By studying these cell lines we found that:

- Constitutive expression of either CRABP II or RARα alone had no significant effect on MDA-MB-231 proliferation or responsiveness to RA. Expression of **both CRABP II and RARα decreased the proliferation of MDA-MB-231 cells.**. Population doubling times of parental MDA-MB-231 cells, two independent RARα expressing, and one CRABP-II expressing MDA-MB-231 cell line were 39, 39 and 41 hours respectively, and these times were unaffected by micromolar retinoic acid. In contrast, the average population doubling time of two independent MDA-MB-231 derived cell lines expressing both RARα and CRABP-II was 108 hours. This increased to ~150 hours in the presence of retinoic acid (10^{-6} M) (Figure 11).

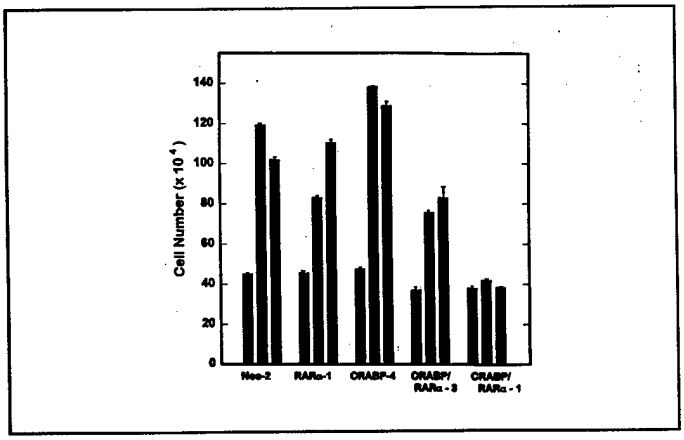


Figure 11. 15 x 10^4 cells were plated on 60 mm dishes and treated with 10^{-6}M RA. Cell numbers and viability were measured on days 0 and 5. Viability was 90+% at all times. Error bars show standard deviations.

Conclusion

The data presented support our hypothesis for a mechanism of RA-induced growth inhibition of HBC cells. Kinetic and quantitative changes in cell cycle progression and gene expression following RA treatment are consistent with RA inducing a block to external mitogenic signals. The earliest measurable effect of RA was repression of the induction of immediate early gene expression, followed by decreased cyclin D1 expression and failure to enter S phase. Our interpretation of these data is that RA induces a state of functional mitogen deprivation in HBC cells. Preliminary data indicate that this effect might involve disruption of PtdIns 3-kinase signaling. By manipulating the expression of CRABP II, RARa and CRBP we have shown that expression of these mediators of retinoid action is sufficient to exert growth inhibitory effects in the presence of serum.

Future work: We have described molecular and biochemical effects of RA which correlate with RA-induced growth arrest of HBC cells. Our goal is to identify in more detail the mitogenic signaling pathways that are disrupted by RA treatment. We have recently demonstrated that RA treatment of serum-starved T-47D cells blocks EGF-induced tyrosine phosphorylation of the EGFR but does not affect EGFR protein level. We are currently investigating the potential role of PKCα in mediating the anti-mitogenic effects of RA, ROL and RAR-selective agonists (Cho et al., 1997). We will perform parallel experiments using T-47D-CRBP cells and MDA-MB-231-RARα-CRABP-II cells to determine the effects of constitutive expression of retinoid binding proteins and receptors on EGF-induced mitogenic signaling. To extend our observations on RA-inhibition of IEG expression, we are currently measuring IEG and AP-1 promoter activation (by conversion of chloramphenicol acetyl transferase) in RA-treated T-47D and MDA-MB-231 cells.

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