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Beta Gene in Human Mammary Epithelial Cells

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13. ABSTRACT (Maximum 200) We have performed β RARE (beta retinoic acid response element) gel shifts and supershifts, using antibodies for RAR α and RAR β , with probes to nuclear extracts from 2 normal and 3 tumor lines. We have performed these analyses with RAR β -transduced breast cancer cells, MCF7 and MDA-MB-231. From 13 breast cancer cell lines, we have PCR-amplified four overlapping regions of the RAR β promoter (totaling ~700 ntds) and performed primary sequence analysis. We have studied the effects of retinoic acid (RA) on growth of normal human mammary epithelial cells (HMECs) in comparison to tumor cells. We have begun experiments to examine the <i>in vivo</i> behavior of breast cancer cell lines transduced with the RAR β gene through nude mice studies. By differential display, we have identified eight genes regulated by retinoic acid in either breast cancer cell lines and/or normal HMECs. We have continued to evaluate the methodology to assess RAR levels in primary breast tumors. Our studies underscore that a rich variety of cellular processes are regulated by RA in concert with RAR β in normal and neoplastic mammary cells.				
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FOREWORD

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Karen Swishelm 9/16/96
PI - Signature Date

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Introduction

Breast Cancer May be Prevented by Retinoids:

Retinoid therapy or dietary intervention may offer new avenues of breast cancer prevention or therapy. Clinical trials in the U.S. (National Cancer Institute) and Italy (Milan) are now determining the efficacy of retinoid chemotherapeutics[1]. The importance of retinoids in the *prevention* of breast cancer is strongly suggested based on the Harvard School of Public Health nurses' cohort study ($n > 89,000$), where it is indicated that moderate dietary intake of carotenoids (plant source of vitamin A) as well as preformed vitamin A is inversely associated with the risk of breast cancer (Rev in: 2). *Note:* this previous study is ongoing and will likely reveal additional trends given longitudinal nature of the study. Additional support for retinoid therapeutics derives from a large body of animal carcinogenesis studies where synthetic retinoids were found to reduce mammary tumor incidence in mice and rats [Rev. in: 3].

Retinoids, including vitamin A and one of its metabolites, all-*trans* retinoic acid (RA), are a class of over 3000 naturally occurring and synthetic compounds. They were first recognized as potential cancer preventive substances 70 years ago when it was observed that epithelium of vitamin A deficient animals resembled that of preneoplastic tissue [4]. Humans obtain retinoids as dietary constituents (usually carotinoids or pre-formed vitamin A) that are metabolized in the intestine, the liver, as well as in "target" tissues such, as the skin, lymphocytes, lung and breast, where it hypothesized they maintain a differentiated phenotype. Esterified retinoids are stored in the liver and may accumulate in organs with high fat content such as the mammary gland [5, 6]. The most widely studied of the retinoid metabolites is retinoic acid (RA). Retinol or precursors of retinoic acid are metabolized *in vivo* to an active metabolite, all-*trans*retinoic acid (AT-RA, often abbreviated to RA). Currently a number of clinical trials are underway using retinoids (natural and synthetic), either alone or in combination with other therapies to treat a variety of cancers, including breast cancer [1]. Our studies of one of the nuclear receptors for retinoic acid, RARB [retinoic acid receptor beta], are based on the paradigm that the action of retinoids in concert with expression of tissue-appropriate wild-type nuclear retinoic acid receptors promotes a differentiated phenotype in breast epithelial cells, and in particular in the luminal epithelial cells where most human breast tumors arise.

Proteins That Participate in Retinoid Action:

Although we are targeting one nuclear RAR for the focus of our studies, we must keep in mind for both our current and future studies the larger picture of retinoid action. Three classes of intracellular proteins may play roles in the phenotypic response of target cells (normal vs. tumor) to retinoids:

- a) the circulating retinol binding proteins and cytosolic retinol and retinoic acid binding proteins [CRBPs; CRABPs]; and
- b) the nuclear, retinoic acid receptors [RARs; RXRs]
- c) the enzymes involved in the transformation of retinol to retinoic acid;

Very little is known about the intracellular proteins which metabolize retinoids and their relationship to cancer risk or prevention. However, it is interesting to note that several of these proteins involved in retinoid uptake and action are themselves regulated at the transcriptional level by retinoic acid, e.g. RAR β and CRBP1.

Much less is known about the expression and regulation of the CRBPs or CRABPs in relationship to breast cancer. A recent study reported expression of CRABP II (but not I) in both RA responsive (growth suppressed) and RA resistant breast cancer cell lines [7]. Interestingly, *in vitro*, both RAR β and CRABP II are upregulated at the mRNA level by retinoic acid. These proteins are likely to be important in sequestering retinol or retinoic acid derivatives and for delivery to subcellular regions (microsomes) where the ligands are metabolized.

A striking number of neoplasias exhibit either direct or indirect involvement of the retinoic acid receptors. The theme in most cases is either a rearrangement in the normal gene or loss of expression. The majority of cases of acute promyelocytic leukemia, for example, exhibit a characteristic translocation between chromosome 15 and 17 (the site for the RAR α gene). Promyelocytic leukemia patients frequently attain remission with retinoids presumably because there is sufficient wild type RAR α in the neoplastic cells to respond and to overcome the transforming, aberrant protein. Recently it was demonstrated that in treatment of patients with "pre-malignant" oral neoplasia with 13-*cis*-retinoic acid, the expression of the RAR β gene was specifically up-regulated and this was associated with "clinical response" [8]

Loss of expression of the retinoic acid receptor *beta* (RAR β) has been documented, to date, in cells derived from the following solid tumors: ovarian cancer [9]; lung cancer [10, 11] squamous cell carcinoma [12] head and neck cancer [13] gastric cancer [14] and breast cancer [15]. This latter paper was work I performed that established, in part, the basis for this Army-funded research. The RAR β gene has properties of a tumor suppressor gene; it slows growth of lung tumors in nude mouse assays [11] and, when transfected, causes breast cancer cells to undergo apoptotic cell death [16, 17]. Growth suppression of breast tumor cells may also be mediated through RAR α and RAR γ [18, 19], although no transfection or *in vivo* experiments strongly support RAR α and RAR γ as tumor or growth suppressors, as is the case for RAR β . There is a strong interest both in academic medicine and in the pharmaceutical industry to design retinoic acid derivatives to target specific receptors and to study these as specific chemotherapeutics specifically for breast cancer (information this P.I. obtained from attendance at a recent FASEB meeting on Retinoids, Copper Mountain, CO., June 23-28, 1996 (also recent refs: 20-22).

Our major, current goals, based on two key papers [15, 16], are to confirm the hypothesis that RAR β is a tumor suppressor gene for breast cancer and to understand the receptor and ligand interactions necessary for invoking and maintaining growth control and/or a differentiated phenotype.

Our broad, future goals include an understanding of the retinoid metabolic pathways, the genes involved in this process, and genes that may transcriptionally modulated by retinoic acid or metabolites of retinoic acid in the mammary gland.

Overview: This progress report addresses four areas of work accomplished over the past year:

1) We have made progress in sequencing and analysis of the RAR β gene promoter (*Task 1*). We have advanced our studies of protein:DNA interactions within the RAR β gene promoter in breast cancer cells (*Task 1*).

2) We have made progress on Task 2 by furthering our understanding of RA growth control in normal HMECs (*see Appendix manuscript*). We have identified an extracellular matrix protein that is regulated by RA in normal cells and RAR β -transduced breast cancer cells MDA-MB-231. We have established the base line studies for animal (nude mouse) experiments.

3) We have made progress in cloning 5 genes by differential display (positive selection by retinoic acid induction in breast tumor cells, MCF7M) and cloned near full length of 2/5. Using the DD technology, we have identified three genes upregulated by retinoic acid in normal human mammary epithelial cells (*Task 3*).

4) We have critically evaluated state of the art methodology to assess levels of RAR β gene expression in primary tumors, and have begun work on *in situ* hybridization and reverse transcriptase- polymerase chain reaction (RT-PCR), using breast tumor cell lines which do or do not express RAR β mRNA (*Task 4*).

Body:

1) Analysis of RAR β promoter (Task 1)

a) **DNA: protein interactions.** We have utilized both the wild type and mutant synthetic, double-stranded BRARE DNA probes, with a direct repeat separated by 5 nucleotides in gel shift assays:

BRAREs	
WT	GGGTAGGGTTCAACGAAAGTTCACTCG
Mutant:	GGGTAGGCTTACCGAAAGTTCACTCG
*= non-identity	* **

And, as an additional negative control, wild type and mutant DNA probes for a consensus **RXRE**, with a direct repeat separated by 1 nucleotide:

RXREs	
WT	CTCTGCAGGTCA CAGGTCACTTTTCCT
Mutant:	CTCTGCACGTACCAGGTCACTTTTCCT
*= non-identity	* **

to test the specificity and sensitivity of DNA:nuclear protein interactions in normal, tumor, and RAR β gene-transduced breast cancer cells [16]. We have also assayed the specificity of the DNA:protein complexes using RAR α -, RXR β - and RAR β -specific antibodies in gel super-shift assays. To address the possibility that other known proteins that "cross talk" with the nuclear retinoic acid receptors are involved in aberrant DNA:protein:protein complexes involving the BRARE in breast tumor cells, we initiated gel shift and supershift assays using AP1 DNA probes and an antibody to the early response/transcription factor, c-jun.

We have tested the following nuclear extracts from cells grown in the presence or absence of RA with ^{32}P -labeled probes:

- 2 normal HMEC strains
- 3 breast cancer cell lines: MCF7, MDA-MB-231, and Hs578T

- 2 series of clones and controls of RAR β -transduced breast cancer cells, MCF7 and MDA-MB-231

Results:

- Both the RAR α and RAR β antibodies caused a supershift in DNA:protein complexes when the target DNA is wild type β RARE. This was observed in both normal and tumor cells
- RXRE complexes are of a lower (less dense) mobility for normal HMECs compared to tumor cells
- MDA-MB-231 cells (parentals, controls, and RAR β -transduced) exhibit diminished binding to the RXRE in cells cultured in RA compared to MCF7 RAR β -transduced cells and controls.
- Both MCF7- and MDA-MB-231- RAR β -transduced cells exhibit abundant supershift complexes specifically with an antibody to RAR β

b) Sequence analysis of the RAR β promoter in breast cancer cell lines: In addition to the three primer sets discussed in last year's progress report, we have utilized an additional primer set (IV).

Primer set I (Region B)*	forward: reverse	5'- AGGAGCAGCGTCCCGGC-3' 5'-CCTACTACTTCTGTCAC-3' inclusive for nucleotides -262 to +16 relative to the start of transcription:
Primer set II (Region A)	forward: reverse	5'- GTGGCCTGTGTGTTTGGGAC-3' 5'-CTCGCAGTGTAGAAATCCAGG-3' inclusive for nucleotides -721 to -464 relative to the start of transcription
Primer set III (Region D)	forward: reverse	5'- GTGGCCTGTGTGTTTGGGAC-3' 5'-CTCGCAGTGTAGAAATCCCAGG-3' inclusive for nucleotides -721 to + 535 relative to the start of transcription
Primer set IV (Region C)	forward: reverse	5'- GTGGCCTGTGTGTTTGGGAC-3' 5'-CCTACTACTTCTGTCAC-3' inclusive for nucleotides -721 to +17 relative to the start of transcription

*See also Appendix I for "regions"

Thus we will be able to compare and verify the sequences of overlapping fragments for each cell line.

We have extracted genomic DNA from 13 breast cancer cell lines and one normal cell line. In the majority of these lines, we extracted DNA from cells received from different sources. For example we have MCF7 cells from ATCC, the Michigan Cancer Foundation, and the Dana-Farber Cancer Institute. Lacking the ability to test differently derived cell sources, we have also obtained DNA from different culture passages in order to be confident that any changes we may see in the sequence of the promoter were likely present in the initially isolated tumor sample or at an early passage in our laboratory. We have designed and

implemented experiments to amplify by PCR each of the four specific regions of the promoter for 33 DNA purifications. PCR amplicons were detected by ethidium bromide staining of 1.8% agarose gels containing electrophoresed- PCR reactions and DNA markers for size determination. DNA bands were excised by a sterile razor blade and purified over a micro concentrator purification column (Amicon). In several instances, two bands were visualized for one PCR, depending on the cell line. In those cases, both bands were excised. Automated fluorescent sequencing was performed by J. Strange, Murdock Laboratory, Univ. of Montana, using one or both of the primers used in the PCR amplification. Currently we are analyzing each region for homology to the published sequence of the RAR β promoter. using a subroutine, Pileup, in GCG (a sequence analysis package, Genetics Computer Group, Madison, WI) on the mainframe computer, McClintock at the University of Washington.

Results:

68 sequences from 13 breast cancer cells are under analysis. Regions of mismatch or gaps are examined on the chromatogram for that sequence. In several cases, we anticipate having to re-amplify and sequence manually. **See Appendix I for an example of a sequence alignment.**

c) Luciferase reporter gene constructs for studying the specific negative regulatory elements utilized by breast tumor cells. We have amplified constructs and verified restriction fragments/inserts for the following RAR β promoter luciferase reporter constructs, all of which were placed in the pGL2 basic vector (Promega):

construct name:	Size of promoter frag. & element(s)*
pL-015	1.655 kb includes TRE, CRE, & β RARE
pL-016	0.902 - includes TRE, CRE, & β RARE
pL-017	0.677 -includes TRE, CRE, & β RARE
pL-018	0.411 -includes TRE, CRE, & β RARE
pL-019	0.398 - includes TRE, CRE, & β RARE
pL-020	0.279 - includes TRE, CRE, & β RARE
pL-021	0.233 - TRE + β RARE
pL-022	0.215 -wt β RARE
pL-023	0.233 - mtTRE + β RARE
pL-025	0.268 - mtCRE + β RARE
pL-026	0.305 - CRE+TRE+mt β RARE
pL-027	0.305 - CRE+mtTRE+mt β RARE

* TRE = thyroid hormone response element; CRE = cAMP-response element; β RARE = retinoic acid response element. All of these elements are found the promoter the RAR β gene. Constructs originated by Hui Tsou, Columbia University.

Following transient transfection with lipofectin (Gibco, BRL), five of these constructs (-015; -018, -019, -020, and -021) have been tested for luciferase activity in RAR β -transduced tumor lines along with positive and negative control plasmids, pGL2 control and pGL2basic. We feel confident that we can perform similar experiments in tumor cell lines.

2) Functional RAR β gene in breast tumor cells and phenotypic changes

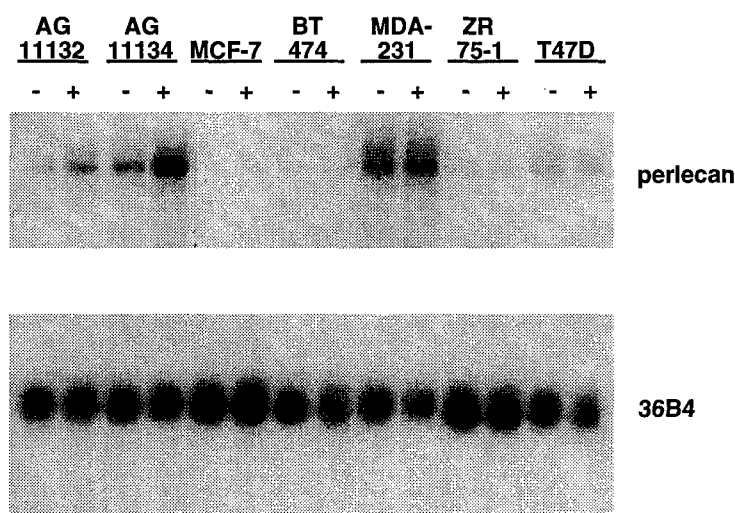
(Task 2): We have introduced the full length human RAR β gene into four breast tumor cell lines: MCF7, MDA-MB-231, MDA-MB-435, and BT 474 (*Cell Growth and Differentiation* 6:1077-1088, September 1995).

We have begun a phenotypic characterization of the RAR β -transduced cells. We observed that the transduced tumor cells expressing RAR β constitutively exhibited a more flattened adherent morphology in culture. We questioned if this was a direct or indirect result of reconstituted RAR β expression. In order to begin to answer this issue, we examined normal HMECs in order to determine if they, too, underwent apoptosis as the RAR β -transduced breast tumor cells did when cultured in RA. We found, in contrast, that normal HMECs failed to undergo apoptosis, but instead underwent a state of growth arrest. This distinguishing result between normal and tumor cells is quite important in light of the possible chemotherapeutic use of retinoid compounds (**see Manuscript, Appendix II**) e.g., if normal cells are preserved, while tumor cells are selectively killed.

We have examined a number (10) of down-stream candidate genes (both oncogenes, cell cycle regulatory genes, and genes that might affect cell morphology) for altered expression as a consequence of the introduction of the RAR β -gene.

*The one candidate that exhibited altered regulatory expression when normal or tumor cells are cultured in RA was the extracellular matrix molecule gene, **perlecan** (see attached, Figure 1, 2 and 3)* Upregulation of perlecan is significant for several aspects of cellular physiology: a) perlecan may serve as a reservoir for positive or negative regulatory growth factors; and b) perlecan deposition is important in maintaining an intact basement membrane [23]. Other investigators have examined perlecan in primary breast cancers and find that more advanced tumor beds exhibited loss of perlecan protein accompanying a loss of intact basement membrane [24].

Figure 1



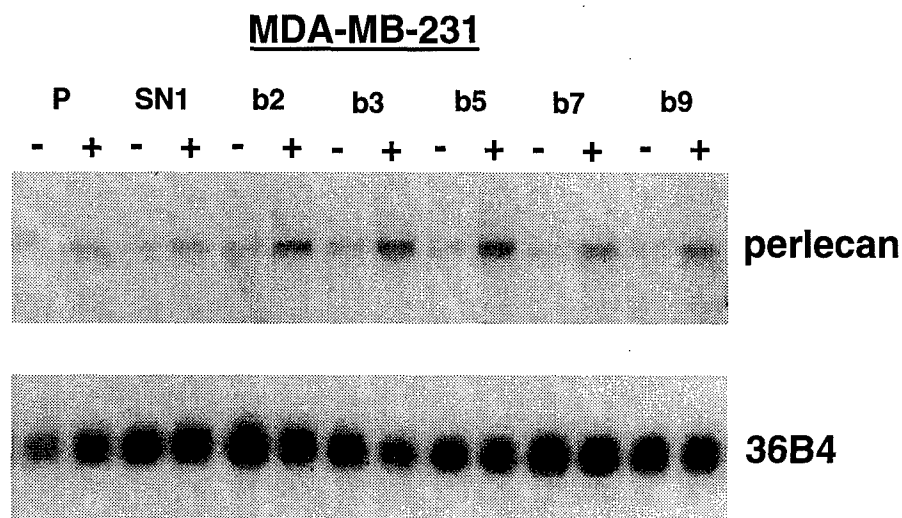
Expression of the perlecan gene in normal and breast cancer cells in response to culture in retinoic acid. Cells were cultured for 48 hours in the absence (-) or presence (+) of 1 μ M retinoic acid. Total RNA was collected and Northern blot analysis was performed. The blot was first probed with a cDNA for perlecan [25] and subsequently with a control probe, 36B4 (see [15, 16] for details of growth, RNA collection and Northern analysis).

Results summary: Perlecan exhibits up-regulation by retinoic acid in two normal HMEC strains, AG-11132 and AG-11134. It lacks appreciable expression in 4 estrogen receptor positive breast tumor cell lines, MCF-7, BT-474, ZR-75-1, and T47D. Perlecan exhibits up regulation in the estrogen receptor negative breast cancer line, MDA-MB-231.

Significance: Elaboration of basement membrane protein perlecan may be down-regulated or altered, particularly in estrogen receptor positive tumors.

Note: this work (and the results shown in Figures 2 and 3) is the result of a collaborative effort among Drs. Michael Kinsella (a proteoglycan expert in the Dept. of Pathology at the University of Washington) and Victoria Seewaldt (Department of Medical Oncology, University of Washington).

Figure 2



Expression of the perlecan gene RAR β -transduced breast cancer cell line, MDA-MB-231. Cells were cultured for 48 hours in the absence (-) or presence (+) of 1 μ M retinoic acid. Total RNA was collected and Northern blot analysis was performed. The blot was first probed with a cDNA for perlecan as described in Figure 1. P = parental line; SN1 = one vector-alone clone; b2, b3, b5, b7, b9 = five independent clones of MDA-MB-231 transduced with a retroviral vector containing the human RAR β gene (normally MDA-MB-231 cells do not express RAR β mRNA).

Results summary: MDA-MB-231 cells transduced with the RAR β gene exhibit enhanced expression (about 10-fold) of the perlecan gene when cells are cultured in RA.

Significance: The basement membrane protein perlecan is a likely downstream gene whose expression is regulated by retinoic acid through transcriptional activation of the RAR β gene. Activation of the perlecan gene is predicted in an *in vivo* model of breast cancer (nude mouse studies).

Figure 3

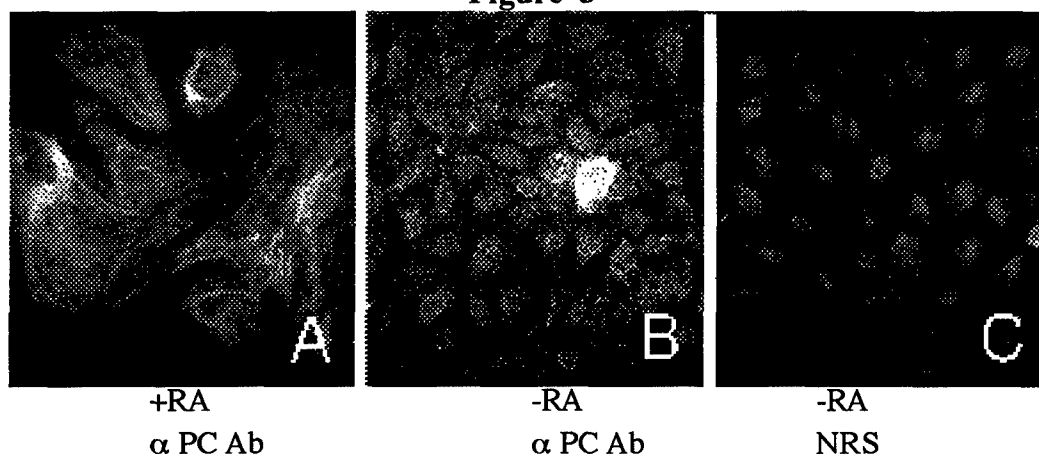


Fig. 3. Perlecan protein deposition is facilitated by RA. Normal HMEC strain, AG-11132 was plated onto slides coated with gelatin and cultured for 3 weeks in the absence of RA. Panel A: HMECs cultured in RA for four days. Panels B and C: HMECs cultured for four days in the absence of RA. All cells were fixed cold in 3% paraformaldehyde, followed by ice cold methanol fixation. Immunocytochemistry was performed with a fluorescence labeled- perlecan antibody [α PCab](1:500 dilution) to cells shown in panels A and B (EY9; rabbit anti-mouse; John Hassell, University of Pennsylvania). Panel C was incubated with normal rabbit serum [NRS].

Results: Normal cells cultured in RA exhibit a distinct alteration in the extracellular matrix as a result of perlecan protein expression, as evidenced by antibody staining *in situ*.

In order to further characterize the RAR β -transduced cells we have initiated nude mouse studies to determine if breast cancer cells that contain the reconstituted RAR β gene show altered tumor growth kinetics *in vivo*. Our first experiments were initiated this summer. We inoculated 4 animals each with 10^6 or 10^5 MDA-MB-435 breast tumor cells as well as sham (media) control into the mammary fat pad of Balb/c nu/nu female mice. We have obtained experimental design advice from Dr. Janet Price, University of Texas, M.D. Anderson Cancer Center, Houston, TX [26]. Our initial experiment was performed in order to determine our success in animal-survival during surgery (100% survived) as well as the time for progression of tumor "take". Animals were sacrificed at 9 weeks and necropsy was performed. We have been very fortunate to have the expert advice and scientific collaboration of investigators from the Comparative Medicine at the University of Washington, Drs. Lillian Price and Ted Birkebak. Dr. Price assisted in the surgeries and in the monitoring of the animals in a specific pathogen free facility. Dr. Birkebak, a veterinary pathologist assisted us in necropsy.

In our next experiment we will test if the RAR β -transduced cells MDA-MB-435 cells exhibit a different biologic course in the nude mouse. We anticipate no difficulties in fulfilling this task.

3) Determination of genes expressed as a consequence of culture in retinoic acid (Task 3): We have continued our studies to detect differences in gene expression in cells cultured in the differentiating agent, retinoic acid. We have used the method of differential display (DD) of mRNA [6] to identify genes regulated by RA. The tables below show our progress in two cell models. The first (Table 1) summarizes our work with MCF7 breast cancer cells; Table 2 summarizes our work with normal HMECs (AG11132 cells). In both models we selected primarily potential mRNAs that were up-

regulated upon RA culture. *In summary, we have 4 genes of known function and , at this time in the sequencing strategy, 4 novel genes that exhibit enhanced transcription with RA.*

Differential Display summary I:

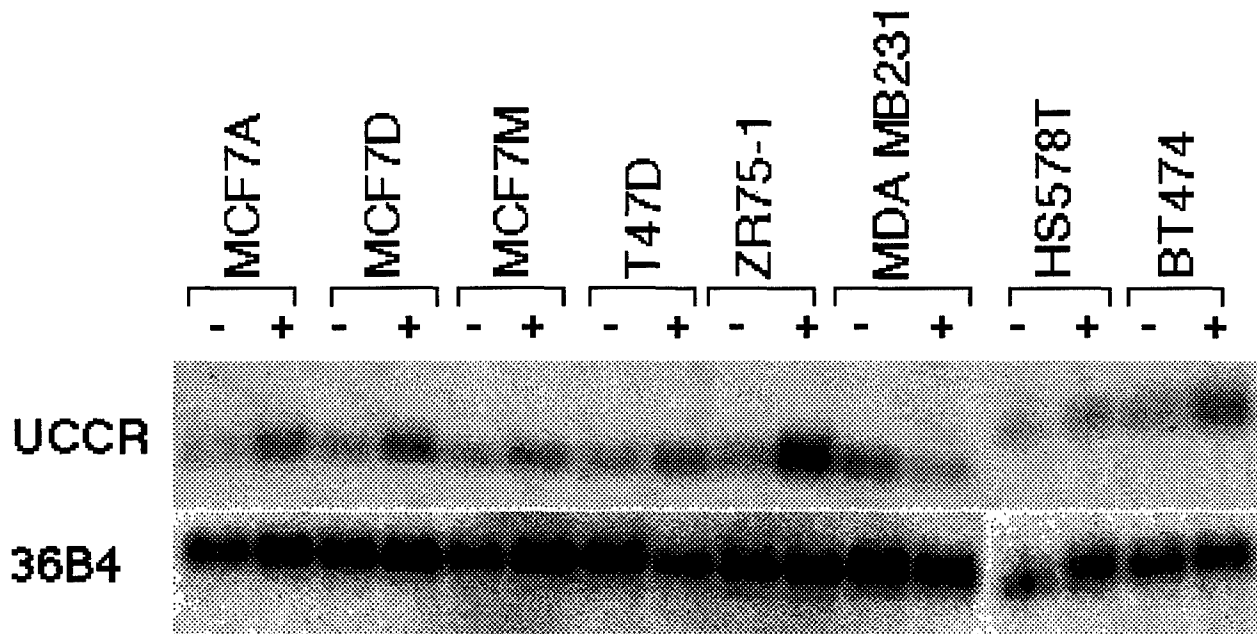
Selection of DD PCR products with RA-associated Enhanced Expression in MCF7 breast cancer cells

Status	Number
Number of primer combinations tested	25
Bands eluted from DD gel	34
Positive candidates on screening Northern	16
Gene candidates cloned + positive on second Northern screen	13
Gene candidates partially sequenced	5
Sequenced candidates for continued studies	5
Candidates for functional studies	1
Candidates to be cloned	1

We have elected to further investigate the functional properties one candidate, which exhibits approximately 4-5-fold increased expression in MCF7 cells cultured in 1 μ M retinoic acid for 48 hours. This gene encodes for a protein related to a ubiquinol-cytochrome C reductase 9.5 kD polypeptide (UCCR). This peptide likely resides in the mitochondria inner space and participates in electron/proton pumping, and ultimately the energy metabolism of cells. We have screened a cDNA library prepared from ZR-75-1 cells (Clontech) and obtained plaques containing various lengths of this gene. The longest clone is ~700 nucleotides, which we believe contains the full-length cDNA sequence, based on the published bovine sequence. The human gene has not been reported. We have begun studies using FACS analysis and mitochondria-specific dyes to ascertain if RA effects electron transport.

Examples of the expression patterns for a sample of these above genes or gene candidates is shown in Figures 4 and 5 below.

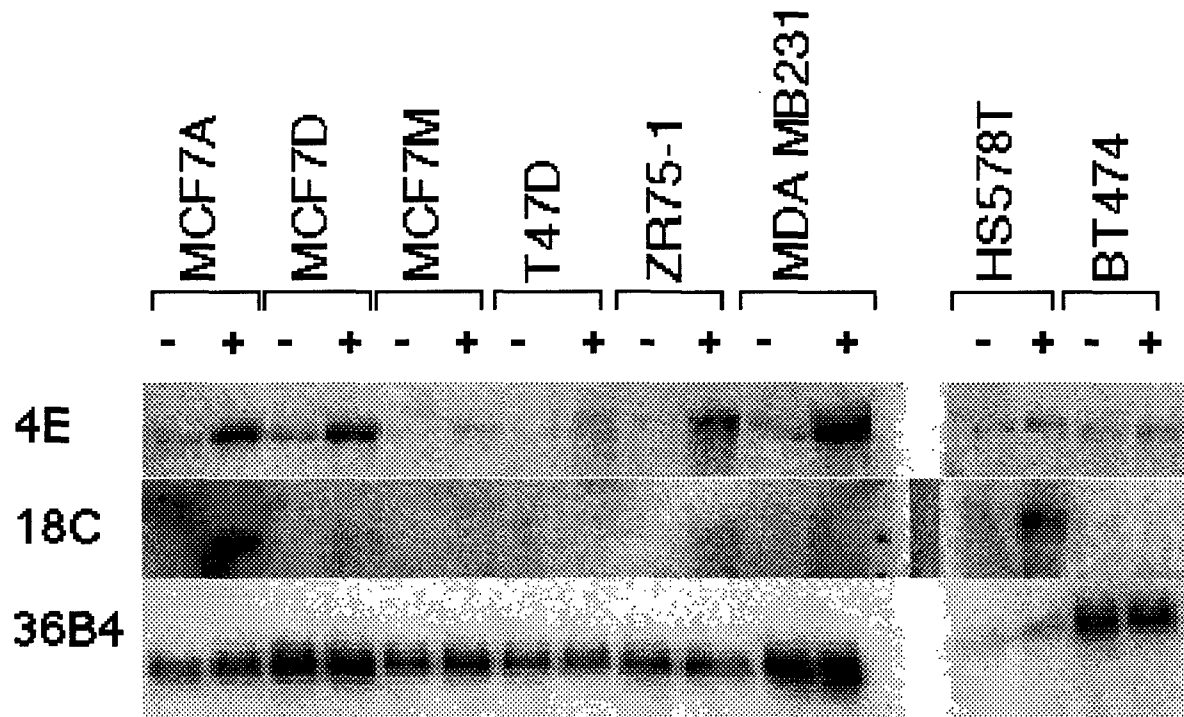
Figure 4



Expression of the human 9.5 kD ubiquinol-cytochrome C reductase (UCCR) gene in breast cancer cells in response to culture in retinoic acid. All cells were cultured for 48 hours in the absence (-) or presence (+) of 1 μ M retinoic acid. As with Figures 1 and 2, total RNA was collected and Northern blot analysis was performed with 10 μ g of total RNA. The blot was first probed with the cloned differential display amplified product and subsequently with a control probe, 36B4 (see previous figures for references on the details of Northern analysis).

Results summary: The expression of the UCCR gene, encoded in the nucleus, with the gene product utilized in complex III of electron transport in the mitochondria, is upregulated in 5/6 breast tumor cell lines. Note: we tested three independent isolates of MCF7, denoted M, D, and A (M = Michigan Cancer Research Foundation; D = Dana-Farber Cancer Institute, Boston; and A = American Type Culture Collection.)

Figure 5



Expression of two genes identified by differential display in breast cancer cells in response to culture in retinoic acid. All cells were cultured for 48 hours in the absence (-) or presence (+) of 1 μ M retinoic acid. As with Figures 1 and 2 and 4, total RNA was collected and Northern blot analysis was performed with 10 μ g of total RNA. The blot was first probed with the cloned differential display (DD) amplified products (4E and 18C) and subsequently with a control probe, 36B4 (see previous figures for references on the details of Northern analysis).

Results summary: The expression results from two cloned DD-PCR products which have been sequenced and found to be novel. Both genes are upregulated by RA in MCF7M cells, and to various extents expressed and upregulated in other breast cancer cell lines. They are both novel in that neither encodes for a known gene, but they both have been cloned and submitted to GenBank as anonymous, EST (expressed sequence tagged) sequences. Note: This is a different blot from Figure 4. We tested three independent isolates of MCF7, denoted M, D, and A (M = Michigan Cancer Research Foundation; D = Dana-Farber Cancer Institute, Boston; and A = American Type Culture Collection.)

Differential Display summary II:

Selection of DD PCR products with RA-associated Enhanced Expression in Normal HMECs, AG11132

Status	Number
Number of primer combinations tested	33
Bands eluted from DD gels	9
Positive candidates on screening Northern	8
Gene candidates cloned	3
Gene candidates partially sequenced	3*
Library screening	N/D

*One candidate encodes a human pre-mRNA splicing factor 2 p32; the second candidate encodes the human dihydrodiol dehydrogenase; and the third shows homology to human clordecane reductase by Blast algorithm searches (World Wide Web databases and GCG).

4) Initiation of studies to determine quantitative and qualitative expression of the RAR β gene in vivo (Task 4).

We have made the least progress on this task during the past year, due primarily to technical difficulties and irreproducibility of experiments, based on the techniques of Ferrari et al. [27]. In situ experiments have been even more challenging. We have plans for specific changes in procedures or tactics.

a) We have been unable to amplify reproducibly the RAR β gene. We are currently revising our strategy for semiquantitative PCR of the RARs from tissue samples, by designing different primers.

b) We have found the RNA:RNA in situ experiments to be problematic in obtaining consistent, repeatable results. We find that tissue from breast biopsy specimens (formalin-fixed and paraffin embedded) to be exquisitely sensitive to Proteinase K digestion. This observation was confirmed to us by Dr. Rubin Lotan's group (discussions at recent FASEB Retinoid meeting).

We have continued to bank breast biopsy specimens and currently have accrued samples from over 140 individuals.

Conclusions:

1. Nuclear extracts from RAR β -transduced breast cancer cells exhibit high levels of protein binding, specifically involving RAR β protein, to the wild type BRARE. We also have demonstrated RAR β -transduced breast cancer cells show differential protein binding to an RXRE consensus. We preliminarily determined that approximately 50% of the tumor cell lines exhibit mutations/deletions in the RAR β promoter. Full confirmation of this preliminary finding will lead us to a more intelligent analysis of potential mutation spectra analysis of the RAR β gene in primary breast tumors.

2. We have demonstrated that one phenotypic sequela of breast cells cultured with retinoic acid is induction of the gene and gene product for perlecan, a heparan sulfate proteoglycan, deposited in the extracellular membrane space.

3. We have identified 9 genes that exhibit enhanced expression in either normal or breast tumor cells when cells are cultured in retinoic acid, identified either by "logic" or by differential display methodology.

Retinoic acid and the nuclear receptor, RAR β , regulate a wide variety of cellular functions and biochemical pathways, from the mitochondria to the extracellular space, in order to maintain cellular homeostasis in mammary epithelial cells.

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Appendix I

Example of primary sequence analysis of the RAR β promoter using the PILEUP algorithm in GCG (Genetics Computer Group).

Hierarchical identifiers include:

- #s 1-24 = coded cell line number
- A, B, C, or D = region of promoter
- F= forward primer
- i or ii = higher molecular weight (i) or lower molecular weight (ii) band excised from PCR gel.

The example shown here does not include the "wild type" RAR β promoter sequence (GenBank identifier X56849).

PileUp of: @total.fil

Symbol comparison table: GenRunData:pileupdna.cmp CompCheck: 6976

GapWeight: 5.000
GapLengthWeight: .300

total.msf MSF: 433 Type: N September 18, 1996 09:16 Check: 3455 ..

Name: 5cCF	Len: 433	Check: 5055	Weight: 1.00
Name: 7cCF	Len: 433	Check: 2057	Weight: 1.00
Name: 14aiAF	Len: 433	Check: 176	Weight: 1.00
Name: 17aiAF	Len: 433	Check: 7737	Weight: 1.00
Name: 13ciCF	Len: 433	Check: 6273	Weight: 1.00
Name: 19CiiF	Len: 433	Check: 117	Weight: 1.00
Name: 19aiAF	Len: 433	Check: 511	Weight: 1.00
Name: 1CCF	Len: 433	Check: 9047	Weight: 1.00
Name: 23CiF	Len: 433	Check: 9818	Weight: 1.00
Name: 21CiF	Len: 433	Check: 9009	Weight: 1.00
Name: 8ciCF	Len: 433	Check: 4028	Weight: 1.00
Name: 2cCF	Len: 433	Check: 770	Weight: 1.00
Name: 3aAF	Len: 433	Check: 5286	Weight: 1.00
Name: 6aAF	Len: 433	Check: 4956	Weight: 1.00
Name: 18aiAF	Len: 433	Check: 9622	Weight: 1.00
Name: 24cCF	Len: 433	Check: 6732	Weight: 1.00
Name: 24aAF	Len: 433	Check: 4428	Weight: 1.00
Name: 22CiF	Len: 433	Check: 2612	Weight: 1.00
Name: 4cCF	Len: 433	Check: 58	Weight: 1.00
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Name: 18bBF	Len: 433	Check: 3610	Weight: 1.00
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Name: 1diDF	Len: 433	Check: 7636	Weight: 1.00
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Name: 15dDF	Len: 433	Check: 2239	Weight: 1.00
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Name: 17aiiAF	Len: 433	Check: 80	Weight: 1.00
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13ciCF .....TG GCAGACTGGA ATTGGAATCT AGTTCAGTTT GATTCCCCAA
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1CCF .....TAG GCAGACTGGA ATTGGAATCT AGTTCAGTTT GATTCCCCAA
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6aAF .....GCNTACTGGA ATAGGAATCT ATNNCACTCT GATNCCCCAC
18aiAF .....TG GCAGACTGG. ATTGGAATCT AGTTCAGTTT GATTCCCCAA
24cCF .cCF.seqTG GCAGACTGGA ATTGGAATCT AGTTCAGTTT GATTCCCCAA
24aAF .....G GCAGACTGGA ATTGGAATCT AGTTCAGTTT GATTCCCCAA
22CiF .....G GCAGACTGGA ATTGGAA. CT AGTTCAGTTT GATTNCCAA
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12ciiCF .....CA TAGCAAGAAT TTAAAGTCCA
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22ciiF .....GCA TAGCAAG. AN TTAAAGTCCA
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18bDF ..... ..CTCGCCC TGCTCATTTA AANANCACTT
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7diDF ..... ..CT CGCTCATTTT AAAAGCNCTT
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16dDF ..... ..CTCGCC TGCTCATTTA AAAAGCACTT
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2dDF ..... ..CTCGCCC AGCTCATTTT ACAAGCACTT
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51

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20ciiF	TTTGATGGTC	GGATGACAGG	G.AGGGGGAA	GAATACAGGA	CACCAGCAGA
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13ciiCF	TTTGATGGTC	GGATGACAGG	G.AGGGGGAA	GAATACAGGA	CACCAGCAGA
17ciiCF	TTTGATGGTC	GGATGACAGG	GCAGGGGGAA	GAATACAGGA	CACCAGCAGA
8ciiCF	TTTGATGGTC	GGATGACNNG	G.AGGGGGAA	TAATACACGA	CACCAACAGA
19DF	TTTGATGGTC	GGATGACAGG	G.AGGGGGAA	GAATACACGA	CACCACCACA
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3bBF	CCNCNCNCC	CNGCCTCTCT	GGNTGTCTGC	GGNGCNNGG	CTGCTTNNAT
24bBF	CNCTCGCTNC	CNGCCTCTCT	GGCTGTCTGC	NNNGCNNGG	CTGCTGGGAT
14bBF	CCCTCGCTNC	CTGCCTCTCT	GGCTGTCTGC	TNNGCNNGG	CTGCTNNGAT
20BF	CCCTCGCTGC	CTGCCTCTCT	GGNTGTCTGC	TNTNGCAGGG	CTGCTTGNAT
12diDF	CGCTCGCTGC	CTGCCTCTCT	GGCTGTCTGC	TTTTGCAGGG	CTGCTGGGAT
13diDF	CGCTCGCTGC	CTGCCTCTCT	GGCTGTCTGC	TTTTGCAGGG	CTGCTGGGAT
18bBF	CGCTCGCTGC	CTGCCTCTCT	GGCTGTCTGC	TNNGCNNGG	CTGCTGGGAT
4diDF	CCCTCGCTGC	CTGCCTCTCT	GGCTGTCTGC	TTTTGCAGGG	CTGCTNGNAT
7diDF	CCNCNCNCTNC	CTGCCTCTCT	GGCTGTCTGC	TTTNGCNNGG	CTGCTGGGAT
1diDF	CGCTCGCTGC	CTGCCTCTCT	GGCTGTCTGC	TTTTGCAGGG	CTGCTGGGAT
5diDF	CNCTCGCTGC	CTGCCTCTCT	GGCTGTCTGC	TTTTGCNNGG	CTGCTGGGAT
22Di	CGCTCGCTGC	CTGCCTCTCT	GGCTGTCTGC	TTTTGCAGGG	CTGCTGGNAT
16dDF	CNCTCGCTGC	CTGCCTCTCT	GGCTGTCTGC	TTTTGCAGGG	CTGCTGGGAT
23DiF	CCCTCGCTGC	CTGCCTCTCT	GGCTGTCTGC	TTTTGCAGGG	CTGCTNGNAT
15dDF	CCCTCGCTNC	CTGCCTCTCT	GGNTGTCTGC	TNTNGCNNGG	CTGCTGGNAN
19BF	CGCTCGCTGC	CTGCCTCTCT	GGCTGTCTGC	TTTTGGAGGG	CTGCTNGGAN
19CiF	CGCTCGCTGC	CTGCCTCTCT	GGCTGTCTGC	TTTTGGAGGG	CTGCTNGGAN
24dDF	CGCTCGCTGC	CTGCCTCTCT	GGCTGTCTGC	TTTTGCAGGG	CTGCTGGGAT
2dDF	CGCTCGCTGC	CTGCCTCTCT	GGCTGTCTGC	NTTGGCAGGG	CTGCTGGGAT
8diDF	CCNCNCNMC	CTNCNTNTCT	GGNTNTCTGC	TANNGCGGGG	NTNCNGNNAT
14aiiAF					
18aiiAF					
17aiiAF					
19aiiAF					
21Diif	ACCNAGNGGG	NNGGGAANCN	AAAAANANNA	NTCTCTGACC	TAGCCGATCT

151

200

5cCF	ACTCTATATG	CTGCATAGAA	CCNGG..ATA	GGATTTTTTCA	NNGTTAATNA
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14aiAF	ACTCTATATG	CTGCATAGAA	CCTGG..AGA	GGATTTTTTCA	AAGTAAATGA
17aiAF	ACTCTATATG	CTGCATAGAA	CCTGG..AGA	GGATTTTTTCA	AAGTAAATGA
13ciCF	ACTCTATATG	CTGCATAGAA	CCTGG..AGA	GGATTTTTTCA	AAGTAAATGA
19Ciif	ACTCTATATG	CTGCATAGAA	CCTGG..AGA	GGATTTTTTCA	AAGTAAATGA
19aiAF	ACTCTATATG	CTGCATAGAA	CCTGG..AGA	GGATTTTTTCA	AAGTAAATGA
1CCF	ACTCTATATG	CTGCATAGAA	CCTGG..AGA	GGATTTTTTCA	AAGTAAATGA
23CiF	ACTCTATATG	CTGCATAGAA	CCTGG..AGA	GGATTTTTTCA	AAGTAAATGA
21CiF	ACTCTATATG	CTGCATAGAA	CCTGG..AGA	GGATTTTTTCA	AAGTAAATGA
8ciCF	ACTCTATATG	CTGCATAGAA	CCTGG..AGA	GGATTTTTTCA	TAGTAAATGA
2cCF	ACTCTATATG	CTGCATAGAA	CCTGG..AGA	GGATTTTTTCA	AAGTAAATGA
3aAF	ACTCTATATG	CTGCATAGAA	CCTGG..AGA	GGATTTTTTCA	AAGTAAATGA
6aAF	ACTCTATATG	CTGCATAGAA	CCTGG..AGA	GGATTTTTTCA	AAGTAAATGA
18aiAF	ACTCTATATG	CTGCATAGAA	CCTGG..AGA	GGATTTTTTCA	AAGTAAATGA

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24aAF ACTCTATATG CTGCATAGAA CCTGG..AGA GGATTTTTTCA AAGTAAATGA
22CiF ACTCTATATG CTGCATAGAA CCTGG..AGA GGATTTTTTCA AAGTAAATGA
4cCF ANTCTATATC CTGCATAAAT CCGNGGATGA GGATTTTNTCA GNGNTAATAA
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15ciiCF TTTCTCAACA TTGCTAGCTT GTGTACAATA TAGGAAAAGT AGGAGGTGAA
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3bBF TTNNTNCGCN CTNNNAGAAN CNNGGGGANTT GGTNATATCA GAGTAGTTGG
24bBF TTNNNANNTCT CTGTGAGAAT CCNNGGGAGTT GGTGATATCA NANTAGTTGG
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16dDF TTTTNNCCT CTGTNANAAT CCNNGGGANTT GGTGATATCA NACTNGTTGG
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15dDF TTTTANCCCT CTGTNAGAAT CCNNGGGAGTT GGTNATATCA NACTAGTNGG
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19CiF TT.....
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14aiiAF .....GGTAA GTGGCAGAGC
18aiiAF .....CTGGTAA GTGGCAGAGC
17aiiAF .....CTGGTAA GTGGCAGAGC
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21DiiF GNTGTGTNCC CACCCACAAC TGGANNANTG TGTNNNNGGA GGTTCCTGGTA

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201

250

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19aiAF ATCTCGAAAG CTGGATTGCA GAGCAAACGA GTGCAGTCAA .TTCAGCCAG
1CCF ATCTCGAAAG CTGGATTGCA GAGCAAACGA GTGCAGTCAA .TTCAGCCAG
23CiF ATCTCGAAAG CTGGATTGCA GAGCAAACGA GTGCAGTCAA .TTCAGCCAG

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21ciF ATCTCGAAAG CTGGATTGCA GAGCAAACGA GTGCAGTCAA .TTCAGCCAG
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6aAF ATCTCGAAAG CTGGATTGCA GAGCAAACGA GTGCAGTCAA .TTCAGCCAG
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15ciiCF GCATATTTGG GGTATGATGA AGTGGAAAAA TAAGAATATT ATGGACTGAA
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20ciiF GCATATTTGG GGTATGATGA AGTGGAAAAA TAAGAATATT ATGGACTGAA
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3bBF GTCNTNNGAA GGTCCNNGN GGGGGTANGG TNCACCNANC N.TCCACTCN
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20BF GTCATTNNAA GGTCCACNCC NCGGGTAGGG NNCACCAAT. ....
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7diDF GTCATTNGAA GGTNCCNCC CNNGGTNNGG TNCACCNANN N.TNCNCTCT
1diDF GTCATTNNAA GGTNACCANC CCGGGTAGGG TNCACCNANN N.TCCNCTCT
5diDF GTCATTTGAN GGTCCNCCNC CNNGGTAGGG TNCACCTANN C.TNCACTCT
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15dDF GTCANTTGAA NGTCNCCANN CCGGGTAGGG TNCACCNAAN N.TCCNCTCT
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19ciF .....
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2dDF GTCATTTGAA GGTAGCACC CCGGGTAGGG TTCACCGAAA GTTTCACTCG
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251

300

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19CiF .....
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2dDF CATATATT.. ...AGGGCAA TTCAATCTTT CATTCGTGT GACAGAAGTA
8diDF CTNTTNNNTT ACGCNTNTCA CNCTCNCTTT AGTCCCGTGN GANGTNACCC
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16ciiCF ACAGAAGTAG TAGAAGGAC .....
15ciiCF ACAGAAGTAG TAGGAAGGAC .....
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7diDF TTAAGAANTG ACCTGTNCAN AGGNAGGANG GTCNAC....
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19ciF .....
24dDF GTANGAAGTG AGCTGTTCAN AGGCNNGAGG GTCTATTCTT TTGCCGNGGG

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18aiiAF					
17aiiAF					
19aiiAF					
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351

400

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17aiAF	AGGCT.TGTT				
13ciCF	AGGGC.TTGC	ATGTGCTTTT	T.CTGGAGT.	GGAAAANTAC	ATAAGTTATA
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1CCF	AGGGC.TTGC	ATGTGCTTTT	T.CTGGAGT.	GGAAAAATAC	ATAAGTTATA
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6aAF					
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24aAF					
22CiF	CCAGGGNCTT	GCNA.....			
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12ciiCF					
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15ciiCF					
23CiiF					
20CiiF					
22CiiF					
13ciiCF					
17ciiCF					
8ciiCF					
19DF					
17bBF					
3bBF					
24bBF					
14bBF					
20BF					
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13diDF	GGGACCACAA	TCCCCCATG	CGATCTGTTT	GAGGACTGGG	ATGCCGAAAA
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4diDF					
7diDF					
1diDF					
5diDF					
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16dDF					
23DiF	GGGACCACAN	NCCCCCATG	CNATCTGTNN	GAGGACTGGG	ATNCCNANAA
15dDF					
19BF					
19CiF					
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8diDF	GAA.....				
4aiiAF					
8aiiAF					
7aiiAF					
9aiiAF					
21Di					

Appendix II

Manuscript submitted

ALL-TRANS-RETINOIC ACID MEDIATES G₁ ARREST BUT NOT APOPTOSIS OF NORMAL HUMAN MAMMARY EPITHELIAL CELLS ¹

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ABSTRACT

Retinoids mediate the normal growth of a variety of epithelial cells and may play an important role in the chemoprevention of certain malignancies. Loss of retinoic acid receptor-beta (RAR β) function may be an important event in mammary carcinogenesis since the majority of breast cancers, in contrast with normal mammary epithelial cells, fail to express this receptor. We previously reported that all-*trans*-retinoic acid (RA) mediates G₁ arrest as well as apoptosis in certain RAR β -transduced breast cancer cell lines. We now report the effect of RA on normal human mammary epithelial cells (HMECs), which express functionally active retinoid receptors. We observe that RA induces growth suppression and G₁ arrest of these HMECs but find no evidence that RA mediates apoptosis in these normal cell strains. This RA-induced G₁ arrest is temporally associated with decreased levels of hyperphosphorylated retinoblastoma protein (pRB) without any significant changes in c-myc, p53, p21, or p27 expression. Our studies suggest that growth inhibition, rather than apoptosis, may be the mechanism by which RA and RA receptors act to prevent the malignant transformation of normal mammary epithelial cells. The molecular target(s) of the activated RA receptors that mediate this G₁ arrest in HMECs appear to be associated with an Rb-dependent pathway.

Running Title: RA-mediated growth inhibition of HMECs

Key words: retinoids, carcinogenesis, cell cycle, mammary epithelial cells, apoptosis, retinoblastoma protein

Footnotes:

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³ Abbreviations: **RAR**, retinoic acid receptor; **RXR**, retinoid X receptor; **RA**, retinoic acid; **HMEC**, human mammary epithelial cells; **RAR β** , retinoic acid receptor-beta; **RARE**, retinoic acid response element; **pRB**, retinoblastoma protein, **cdk**, cyclin dependent kinase, **TdT**, terminal deoxynucleotidyl transferase; **PBS**, phosphate buffered saline; **ECL**, enhanced chemiluminescent detection; **DAPI**, 4',6-diamido-2-phenylindole; **FACS**, fluorescence activated cell sorting; **RT**, room temperature; **DMSO**, dimethyl sulfoxide; **T.E.**, Tris-ethylenediaminetetraacetic acid; **CAT**, chloramphenicol acetyl transferase.

INTRODUCTION

Vitamin A (retinol) and its derivatives (retinoids) support the normal growth and differentiation of epithelial cells (1, 2). Retinoids are also effective in the prevention of many human malignancies (3-13) and currently there are ongoing clinical trials to test the ability of a synthetic retinoid, N-(4-hydroxyphenyl)retinamide [Fenretinide], to prevent contralateral breast cancer (14, 15). In order to use retinoids in the most clinically beneficial manner, it is important to understand the molecular basis of activity. The actions of retinoids are ultimately thought to be mediated through specific nuclear retinoic acid receptors (RARs)³ and retinoid X receptors (RXRs) belonging to the steroid/thyroid superfamily of transcription factors (16-20). RAR α is expressed ubiquitously in adult tissue and RAR γ is primarily expressed in skin. RAR β is unique because it is primarily expressed in epithelial cells and exhibits induced expression in response to retinoic acid mediated by an enhancer element found in its promoter, the retinoic acid response element (RARE) (21, 22).

Since retinoic acid receptors appear to play a key role in mediating retinoid action, it is likely that these receptors also regulate the anticarcinogenic actions of retinoids. This paradigm is consistent with the finding that while RAR β is primarily expressed in many normal epithelial derived-cells or tissues, it is not expressed in a majority of epithelial tumors or in tumor cell lines (23-28). It has been observed that normal human cultured mammary epithelial cells (HMECs) express RAR β mRNA; in comparison, most breast cancer cells fail to express this gene (23, 29, 30). Furthermore, RAR β mRNA expression is upregulated in senescent HMECs (23). This suggests that loss of retinoic acid receptor function may be an important event in solid tumor carcinogenesis.

We previously developed an *in vitro* system to investigate how retinoic acid receptors may

act to prevent the malignant transformation of human mammary epithelial cells (24). The human breast cancer cell lines MCF-7 and MDA-MB-231 do not normally express RAR β and are resistant to RA-mediated growth inhibition at 1.0 μ M all-*trans*-retinoic acid (RA). By retroviral mediated gene transfer, we constitutively expressed the human RAR β gene in both of these breast cancer cell lines and demonstrated that RAR β -transduced MCF-7 and MDA-MB-231 cells readily undergo growth inhibition when treated with RA (24). In addition, the RAR β -transduced MCF-7 cells undergo apoptosis after 4 days of treatment with RA (24). This suggests that RA and RAR β are important mediators of proliferation in these breast cancer cell lines and provides insight into how RA might act to prevent cancer in normal epithelial cells. If the mechanism of action of retinoids and retinoic acid receptors in normal human mammary epithelial cells is to regulate proliferation and apoptosis, then loss of retinoid receptor function might disrupt an important mechanism of maintaining normal tissue homeostasis and thereby contribute to malignant transformation.

In this report we investigate the growth regulation of normal mammary epithelial cells (HMECs) by retinoic acid. HMECs are growth factor-dependent cells derived from reduction mammaplasty specimens and exhibit a limited *in vitro* lifespan. We observe that RA-treated HMECs in culture undergo growth inhibition predominantly associated with G₁ arrest but they do not undergo apoptosis. While p53, p21, and c-myc levels remain constant in HMECs which have undergone this RA-mediated G₁ arrest, there is a marked reduction in the levels of hyperphosphorylated pRB. This is consistent with previous observations in RA-treated T-47D breast cancer cells (31). Our results suggest that retinoids and retinoic acid receptors may be important mediators of proliferation in HMECs but do not play an important role in mediating apoptosis in these cells.

RESULTS

NORMAL HUMAN MAMMARY EPITHELIAL CELLS (HMECS) EXPRESS RA-INDUCIBLE RAR β MRNA

We determined the levels of retinoic acid receptor- α (RAR α) and retinoic acid receptor- β (RAR β) mRNA in normal human mammary epithelial cell strains AG11132 and AG11134 with and without treatment with 1 μ M RA for 48 hours. The expected 3.6 and 2.8 kb RAR α messages were observed in both RA-treated and untreated HMECs. AG11132 RAR α mRNA levels were slightly increased by treatment with RA. In contrast, RAR α mRNA levels increased three fold in AG11134 after RA-treatment (Figure 1). Unlike most breast cancer cell lines (23, 24), HMEC strains AG11132 and AG11134 express RAR β mRNA, albeit at low levels relative to RAR α . RAR β mRNA levels increased 2- and 4- fold respectively for AG11132 and AG11134 after treatment with 1 μ M RA (Figure 1). These HMEC strains also expressed RXR β (at levels comparable to RAR α) and low levels of RAR γ mRNA (data not shown).

RA MEDIATED *TRANS*-ACTIVATION IN CULTURED HMECS

Since HMEC strains exhibit RA-inducible RAR β expression (Figure 1), we wished to determine whether the retinoid receptors expressed in these HMEC strains are functional and able to *trans*-activate a retinoic acid response element in response to RA. We performed transient expression assays utilizing the pRRE4-tkCAT reporter plasmid which contains the natural RA-response element (RARE) from the promotor region of the human RAR β gene (22). Retinoid receptor functional activity was demonstrated in HMEC strains AG11132 and AG11134 by induction of RA-mediated *trans*-activation with increasing

concentrations of RA (Figure 2).

GROWTH INHIBITION AND CELL CYCLE CHANGES OBSERVED IN NORMAL HUMAN MAMMARY EPITHELIAL CELL STRAINS IN RESPONSE TO RETINOIC ACID

HMEC strains AG11132 and AG11134 were cultured in 0, 0.1, 1.0, or 10 μ M RA. We observed RA-mediated growth inhibition of HMECs that was both dose and time dependent. Increased growth inhibition was demonstrated with increasing concentrations of RA and increasing time of exposure in both AG11132 and AG11134 (Figure 3). Growth inhibition after treatment with 1.0 μ M RA was observed starting at 24 hours. Ten micromolar RA appeared cytotoxic to HMECs. These data demonstrate that RA inhibits the proliferation of normal mammary epithelial cells in culture.

In order to determine more precisely the mechanism by which retinoic acid inhibits the proliferation of HMECs, FACS analysis was performed on AG11132 and AG11134 cells treated with 0, 0.1, and 1.0 μ M RA for 48 hours. HMECs treated with 0.1 or 1.0 μ M RA for 48 hours exhibited an increase in the percentage of cells in G_1 and a decrease in the percentage of cells in S-phase relative to untreated controls (Table 1). After 48 hour of treatment with 1.0 μ M RA, AG11132 and AG11134 cells, respectively, exhibit a 17% and 13% increase in G_1 and a 58% and 68% decrease in S-phase. These results demonstrate that RA may inhibit proliferation of HMECs primarily by inducing G_1 arrest.

The effects of 1.0 μ M RA on HMEC cell cycle phase distribution were examined over a defined time course to investigate the kinetics of RA-mediated G_1 arrest. Figures 4 A and B shows that both AG11132 and AG11134 cell strains undergo growth inhibition starting 24 hours after treatment with RA. After 48 hours both cell strains exhibited a greater than

50% reduction in the percentage of cells in S-phase and a corresponding increase in the number of cells in G₁.

ALL-TRANS-RETINOIC ACID DOES NOT INDUCE APOPTOSIS IN RA-TREATED HMECS

We previously reported that MCF-7 cells transduced with RAR β underwent apoptosis when treated with RA (24). In contrast, MDA-MB-231 cells transduced with RAR β underwent G₁ arrest but did not undergo apoptosis when treated with RA (24). In order to determine whether RA mediates apoptosis in normal human mammary epithelial cells, we treated HMEC strains AG11132 and AG11134 with 1 μ M all-*trans*-retinoic acid and investigated whether apoptosis was observed by morphologic criteria and by biochemical parameters.

Morphologic changes characteristic of apoptosis include nuclear condensation, loss of adherence, and cell shrinkage (33, 34). HMECs treated with 1 μ M RA did not exhibit these morphologic changes by either light or electron microscopy (data not shown). HMECs treated with 1 μ M RA for 5 days did not demonstrate nuclear condensation by fluorescent staining (Figure 5).

Internucleosomal DNA fragmentation is characteristic of apoptosis and distinguishes it from other modes of cell death such as necrosis (35, 36). MCF7-RAR β transduced cells undergoing RA-mediated apoptosis demonstrate increased DNA fragmentation starting 4 days after treatment with RA (24). In contrast, we did not observe increased fragmented cytoplasmic DNA by the diphenylamine assay 2, 4, or 6 days after treatment of HMECs with 1 μ M RA (Figure 6). Moreover, ethidium bromide-stained DNA extracted from RA-treated HMECs did not demonstrate DNA laddering following two, four, or six days

treatment with 1 μ M RA (data not shown). Finally, apoptotic strand breaks were not detected by the terminal deoxynucleotidyl transferase (TdT) method in HMECs after 5 days treatment with 1 μ M RA (data not shown). These together data suggest that while RA induces growth arrest of two independent normal mammary epithelial strains in culture, RA does not induce apoptosis in either of these cell strains.

RA-TREATED HMECS DEMONSTRATE A REDUCTION IN THE LEVELS OF HYPERPHOSPHORYLATED pRB

We performed Northern and Western analysis to determine whether the RA-mediated growth arrest observed in RA-treated HMECs was associated with a change in expression of specific genes important in growth control and proliferation of epithelial cells, including c-myc, p53, p21, p27, and pRB (37-56).

The c-myc proto-oncogene is a transcription factor that participates in the opposing cellular fates of proliferation and apoptosis. We investigated the expression of c-myc mRNA expression in normal mammary epithelial cells strains AG11132 and AG11134 which do undergo G₁ arrest and observed that neither HMEC cell strain demonstrates a change in c-myc mRNA expression after 48 of treatment with RA (Figure 7).

The tumor suppressor protein pRB appears to play a critical role in mediating cell cycle progression (48-56). We observe that RA-mediated growth inhibition in HMEC strains AG11132 and AG11134 is temporally associated with a significant decrease in the level of hyperphosphorylated pRB (Figure 8 and 9). We observe that both HMEC strains undergo G₁ arrest starting at 24 hours following retinoic acid treatment (Figure 4). Concomitant with this observed growth arrest, the levels of hyperphosphorylated pRB begin to decline

after 24 hours of treatment with RA (Figure 9). After 48 hours the levels of hyperphosphorylated pRB are reduced by 50% and 63% for AG11132 and AG11134, respectively (Figure 9). These observations suggest that inhibition of pRB hyperphosphorylation is temporally associated with the RA-induced G₁ arrest in HMECs.

To determine whether this RA-induced hypophosphorylation of pRB was associated with any of the known inhibitors of cyclin dependent kinase activity, we compared the relative expression of p53, p21/WAF1/CIP1, and p27 in untreated and RA-treated HMEC strains. We observe that AG11132 and AG11134 HMEC cells strains treated with 1.0 μ M RA for 24 hours (data not shown) or 48 hours (Figure 8) do not exhibit increased p53 or p21 protein levels associated with this RA-induced G₁/S arrest. Both AG11132 and AG11134 HMEC strains express low levels of p27 mRNA (Figure 7), but neither cell strain express detectable levels of p27 protein before or after RA-treatment for 24 or 48 hours (data not shown).

DISCUSSION

The association between vitamin A deficiency and the development of cancer suggests that retinoid-dependent signaling pathways have a role in the suppression of carcinogenesis (2). Therefore, for the effective clinical use of retinoids, it is important to define how retinoid action is mediated in both malignant and normal mammary epithelial cells. The retinoic acid receptor-beta (RAR β) is primarily expressed in epithelial cells and appears to be a critical mediator of retinoid action (23, 24). While RAR β is expressed in normal epithelial tissues, its expression is selectively lost in many epithelial tumors suggesting that the loss of RAR β expression may be a key event in solid tumor carcinogenesis (23-28). It has recently been reported that RAR β expression is selectively lost in patients with oral dysplasia and that a

clinical response to 13-*cis*-retinoic acid was associated with restoration of RAR β expression (57).

In previous studies, we investigated how RA and RAR β might act in breast cancer cells by utilizing retroviral-mediated gene transfer to introduce RAR β into breast cancer cell lines which do not normally express RAR β (24). We observed that while MCF-7 and MDA-MB-231 breast cancer cell lines are resistant to RA-mediated growth inhibition, RA induces G₁ arrest in these two breast cancer cell lines when they are transduced with RAR β . Moreover, RA induces apoptosis in RAR β -transduced MCF-7 cells but not in RAR β -transduced MDA-MB-231 (24). A recent report by Liu *et al.* (58) supports these findings by demonstrating that RAR β mediates the growth inhibitory effects of retinoic acid by promoting apoptosis in human breast cancer cell lines. Furthermore, there is evidence that retinoids and RAR β may mediate apoptosis in other cell types since RA-mediated truncation defects of the embryonic limb appear to be the result of RAR β -mediated apoptosis (59). Based on such observations we hypothesized that retinoids and the retinoic acid receptors could participate in G₁ arrest and apoptosis in normal human mammary epithelial cells.

The normal mammary epithelial cell strains, AG11132 and AG11134, described in this study are derived from reduction mammoplasty specimens and consist of a heterogeneous population of growth factor dependent epithelial-derived mammary cells. Unlike immortal cell lines which retain characteristics of normal cells such as MCF-10A (60), HMEC strains AG11132 and AG11134 are not immortal. In addition, while most breast cancer cell lines do not express RAR β , these HMEC strains express RA-inducible RAR β mRNA (Figure 1). Moreover, the retinoid receptors in these HMEC strains demonstrate normal functional activity as evidenced by RA-mediated *trans*-activation of a RARE driven CAT reporter construct (Figure 2). We wished to determine whether RA might induce growth arrest and/or apoptosis of these HMECs. We observe that RA inhibits proliferation of HMECs

and this is associated primarily with G₁ arrest (Table 1, Figure 4) rather than apoptosis (Figures 5 and 6). In contrast to previous observations in RAR β -transduced breast cancer cell lines (24, 58), we do not observe that RA mediates apoptosis in these two HMEC strains. The absence of apoptosis is confirmed by the lack of characteristic morphologic changes by both light and electron microscopy, and the absence of fragmented DNA (Figures 5 and 6). These data indicate that RA and retinoid receptors mediate G₁ arrest but not apoptosis in normal human mammary epithelial cells.

The observations described in this report may provide a potential model for how RA may act to prevent malignant transformation of normal mammary epithelial cells. If a critical function of RA and retinoic acid receptors is to regulate proliferation, then loss of retinoid receptor function might result in dysregulated growth of human mammary epithelial cells. Further mutations of tumor suppressor genes or oncogenes in this expanded cell population could result in the progression to overt malignancy. This model predicts that loss of retinoid receptor expression and/or function is likely to be an early event in the multistage development of breast cancer in normal mammary epithelial cells.

Growth factors are thought to regulate transit from G₁ to S-phase in almost all cell types but the specific growth factor requirements of mammary epithelial cells are only partially understood. In this report we provide evidence that the differentiating agent RA may play an important role in regulating the G₁ to S-phase cell cycle transition in normal human mammary epithelial cells. Our data suggest that G₁ arrest in HMECs is temporally associated with a decrease in the levels of hyperphosphorylated pRB (Figure 8) in the absence of change in the expression of p53, p21, or p27 protein (Figures 7 and 8) or the expression of c-myc mRNA (Figure 7).

The c-myc proto-oncogene is a transcription factor that participates in the opposing cellular

fates of proliferation and apoptosis. We previously investigated the expression of c-myc mRNA expression in RA-treated RAR β transduced breast cancer cell lines and vector controls and observed that the RA-mediated growth arrest of MDA231-RAR β transduced cells is associated with c-myc down regulation relative to controls (after 48 hours treatment) whereas, the RA-induced apoptosis of MCF7-RAR β transduced cells is not associated with c-myc down regulation (24). From these observations we hypothesized that the lack of c-myc down regulation noted in the RA-treated MCF7-RAR β transduced cells might be related to their ability to undergo RA-mediated apoptosis. We likewise investigated the expression of c-myc mRNA expression in normal mammary epithelial cells strains AG11132 and AG11134 which do undergo G₁ arrest but not apoptosis. Unlike MDA-MB-231-RAR β transduced cells, we observe that neither HMEC cell strain demonstrates a decrease in c-myc mRNA expression after 48 of treatment with RA (Figure 7).

The tumor suppressor protein pRB appears to play a critical role in mediating cell cycle progression. The molecular basis cell cycle progression through the late G₁ restriction point is not fully understood but it appears to correlate with the hyperphosphorylation of pRB. pRB is underphosphorylated throughout G₁ phase, phosphorylated just prior to cells entering S-phase, and remains phosphorylated until late mitosis (48-55). Mutations in Rb are implicated in cell cycle dysregulation and appear to be important in the genesis of a wide variety of cancers (56). We observe that RA-mediated growth inhibition in HMEC strains AG11132 and AG11134 is temporally associated with a significant decrease in the level of hyperphosphorylated pRB (Figure 8 and 9). We observe that both HMEC strains undergo G₁ arrest starting at 24 hours following retinoic acid treatment (Figure 4). Concomitant with this observed growth arrest, the levels of hyperphosphorylated pRB begin to decline after 24 hours of treatment with RA (Figure 9) indicating that inhibition of pRB phosphorylation is temporally associated with RA-induced G₁ arrest in HMECs.

Radiation and other DNA-damaging agents have been found to block pRB hyperphosphorylation. The cell cycle checkpoint gene p53 appears to play an important role in mediating G₁ arrest induced by these agents (37,38). Current models suggest that when cells are exposed to DNA damaging agents such as radiation or chemotherapy, p53 expression is induced. Elevated p53 levels in turn lead to induction of the cyclin inhibitor p21/WAF1/CIP1 resulting in G₁/S arrest associated with pRB hypophosphorylation (39-44). The mechanism(s) by which differentiating agents such as RA might mediate G₁/S arrest may be different than those observed for DNA-damaging agents. We did not observe any increases in either p53 or p21 protein expression when AG11132 and AG11134 HMEC cell strains were treated with RA (Figure 8). Thus the G₁/S growth arrest noted in RA-treated HMECs may occur through a p53 and p21-independent pathway.

The potential link between retinoids and pRB may provide a clue as to how loss of retinoid receptor function in normal mammary epithelial cells might promote breast cancer carcinogenesis. pRB is felt to regulate cell cycle proliferation by association/dissociation with transcription factors, such as E2F (61). Recent data suggests that pRB may regulate more aspects of the cell cycle than previously suspected. Data presented by Cavanaugh *et al.* (62), suggest that pRB might regulate ribosomal RNA transcription mediated by Pol I. This model, that pRB might be a more general regulator of transcription needed for cell proliferation is supported by a recent report by White *et al.* (63), demonstrating that pRB represses a majority of genes transcribed by Pol III in cultured primary mouse fibroblasts. It is currently not known whether pRB hyperphosphorylation in late G₁ causes an increase in Pol-III-mediated transcription.

The question remains whether the observed decrease in hyperphosphorylated pRB is mediated through inhibition of cyclin/cdk complexes or is regulated through other

mechanisms. We are currently investigating the expression of specific cyclins and their respective cyclin dependent kinases, such as cyclin D1/cdk4 and cyclin E/cdk2, felt to be important in phosphorylation of pRB. In a previous report by Wilcken *et al.* (31), RA-treatment did not have an effect on the protein levels of cyclins D1 or E and cdk2 or cdk4 in the breast cancer cell line T47-D. Furthermore, while decreases were observed in cdk2 and cdk4 kinase activity in RA-treated T47-D cells, these changes were observed only after a decrease in the level of pRB phosphorylation occurred (31). These observations, as well as those presented here, suggest that growth regulation by the differentiating agent, RA may be manifest through a pRB-dependent pathway but the precise mechanism remains to be elucidated.

MATERIALS AND METHODS

MATERIALS

All-*trans*-retinoic acid (Sigma) 1 mM stock solution was prepared in 100% ethanol and stored in opaque tubes at -70°C. Control cultures received equivalent volumes of ethanol. Retinoic acid stocks were used under reduced light.

CELL LINES AND MEDIA

Normal human mammary epithelial cell (HMEC) strains AG11132 (M. Stampfer #172R/AA7) and AG11134 (M. Stampfer #48R/AC170) were purchased from the National Institute of Aging, Aging Cell Culture Repository (Coriell Institute) (32). AG11132 and AG11134 strains are cultivated from normal tissue obtained at reduction mammoplasty. These normal cell strains have a limited life span in culture and fail to divide after approximately 20 passages. AG11132 was at passage 8 and AG11134 was at passage 6 at the time of receipt. HMECs are grown in Mammary Epithelial Cell Basal Medium (Clonetics) supplemented with bovine pituitary extract (Clonetics #CC4009) 4 µl/ml, insulin (UBI) 5 µg/ml, epidermal growth factor (UBI) 10 ng/ml, hydrocortisone (Sigma) 0.5 µg/ml, isoproterenol (Sigma) 10 µM, HEPES buffer (Sigma) 10 mM [standard medium]. Cells were cultured at 37° C in a humidified incubator with 5% CO₂/95% air. We did not process our growth media to remove endogenous retinoids. Mycoplasma testing was performed as previously reported by Russell (64).

NORTHERN BLOTTING

RNA was extracted with guanidine isothiocyanate and subjected to Northern blotting in formaldehyde denaturing gels as previously described (23). Ten micrograms of RNA were loaded per lane. Molecular probes utilized in the Northern analysis are as follows: The

hRAR α probe is a 1.3 kb, SmaI fragment (17). The hRAR β probe is a 1.4 kb SacI, BamHI fragment (17). The human c-myc probe is a 1.8-kb EcoRI fragment (24). The p27 probe is a 900 bp fragment (gift of Jim Roberts). The 36B4 probe (700bp PstI fragment) (65) was used as a loading and transfer control probe.

CAT REPORTER ASSAY

HMECs were plated in T-75 tissue culture flasks (Corning) in standard media 24 hours prior to transfection and were approximately 50% confluent at the time of transfection. Cells were transfected by CellFECTINtm (Gibco/BRL Life Technologies) as per the manufacturer's recommendations for transient transfection of adherent cells. Transient transfections were performed utilizing the pRRE4-tkCAT reporter plasmid which contains four copies of the natural RA-response element (RARE) present in the promotor region of the human RAR β gene (22). Transfection control was provided by the pCMV-GH plasmid (34). Transfection conditions were as follows: 10 μ l CellFECTINtm in 1.0 ml standard medium was added to 1.0 ml of standard medium containing 10 μ g of pRRE4-tkCAT reporter plasmid (24) and 10 μ g of pCMV-GH (32), incubated for 10 mins at room temperature, and then added per T-75 flask. Twenty-four hours after transfection, the cells were washed with PBS and re-fed with standard medium containing 0, 0.1, 1.0, or 10 μ M RA. Control cultures received an equivalent volume of ethanol (0.1%). After 24 hours, culture media was collected for determination of growth hormone concentration using a radioimmunoassay kit (Nichols Institute). Preparation of cell lysates and CAT assays were performed according to published methods (66). Twenty microliters of cellular extract was added to 80 microliters of CAT reaction mixture [final concentration: 0.1 M Tris pH 7.8, 2.0 nM n-butyl Coenzyme A (Sigma), 0.15 μ Ci [¹⁴C]-labeled chloramphenicol (Amersham)] and incubated for 3 hours at 37°C. The reaction mixture was extracted with 200 μ l xylenes, vortexed 30 seconds, and centrifuged at 4,000 rpm for 5 min. One hundred seventy microliters of the upper phase was transferred to a new Eppendorf tube

and extracted with 85 μ l T.E. [10 mM Tris (pH 8.0), 1 mM ethylenediaminetetraacetic acid]. One hundred forty microliters of the upper phase was transferred to a scintillation vial and counted. Protein concentration of cell lysates were determined by a Pierce bicinchoninic acid commercial kit. CAT counts were normalized for transfection efficiency as determined by the growth hormone reporter internal control and total protein. Controls were performed to insure that the pCMV-GH plasmid did not demonstrate increased growth hormone activity in response to retinoic acid.

CELL GROWTH CURVES

HMECs were plated in duplicate at 1×10^4 per well in 24 well plates (Corning) and grown in standard medium with 0, 0.1, or 1.0 μ M RA. Untreated controls received an equivalent volume of ethanol (0.1%). Cells were trypsinized at 12 to 24 hour time intervals and counted in triplicate. Cultures did not exceed ~70% confluency.

DNA STAINING OF CELL NUCLEI WITH PROPIDIUM IODIDE AND FACS SCAN ANALYSIS

5×10^5 cells were plated in T75 flasks (Corning) and grown in standard medium with 0, 0.1, 1.0, or 10 μ M RA. Untreated controls received an equivalent volume of ethanol (0.1%). Cells were harvested at 2 days and did not exceed ~70% confluency. For the RA time course: 5×10^5 cells were plated in T75 flasks on day -1 and allowed to adhere. On day 0 the medium was removed and replaced by 20 ml of fresh medium. RA was added to the culture medium for a final concentration of 1 μ M on days 0, 1, 2, and 3 for the preparation of day 4, 3, 2, and 1 time points respectively. Cells were harvested on day 4 and did not exceed ~70% confluency. Preparation of cells for FACS analysis has been previously described (24). Ten thousand events were collected in list mode fashion, stored, and analyzed on Multicycle AV software (Phoenix Flow Systems).

NUCLEAR STAINING OF RA-TREATED HMECS

1×10^5 cells were plated in T75 flasks (Corning) and grown in standard medium with or without $1.0 \mu\text{M}$ RA. Untreated controls received an equivalent volume of ethanol (0.1%). Cells were harvested at 5 days and did not exceed ~70% confluency, were trypsinized and washed once in cold PBS (pH 7.4), and were fixed in 1% formaldehyde in PBS for 15 mins on ice and washed once with cold PBS. Cells were resuspended in 70% ice cold ethanol and immediately transferred to -20°C . Cytospin preparations utilized 1×10^4 prepared cells, spun at 400 rpm for 10 mins in a Cytospin 3 centrifuge (Shandon). Cytospin preparations were stained with 4',6-diamido-2-phenylindole (DAPI) solution [$10 \mu\text{g/ml}$ DAPI in 10% dimethyl sulfoxide (DMSO), 0.85% NaCl, 10 mM Tris (pH 7.4), 5 mM CaCl_2 , 20 mM MgCl_2 , and 0.5% bovine serum albumin] for 10 minutes and then washed three times with PBS. Fluorescence was analyzed utilizing a Axiophot microscope (Zeiss).

ELECTRON MICROSCOPY

1×10^4 cells were plated per well in 6 well plates (Corning) and allowed to attach in standard medium. Retinoic acid stock was added after 24 hours for a final concentration of $1 \mu\text{M}$. After 5 days exposure to RA, cells were then fixed in half-strength Karnovsky's fixative (67) for 6 hours, rinsed in 0.1 M sodium cacodylate buffer and post-fixed in 1% collidine-buffered osmium tetroxide. Dehydration in graded ethanol and propylene oxide was followed by infiltration and embedding in Epon 812. Approximately 70-90 nm sections were stained using saturated aqueous uranyl acetate and lead tartrate. Photographs were taken using a JEOL 100 SX transmission electron microscope operating at 80 kV.

DIPHENYLAMINE ASSAY FOR DETERMINATION OF CYTOPLASMIC DNA FRACTION

2.5 to 5.0×10^5 cells were plated per T25 flask (Corning) and grown in standard media. Retinoic acid stock was added on days 0, 2, and 4, directly to the media to bring the final concentration to $1 \mu\text{M}$. Confluency did not exceed ~70%. On day 6 cells were trypsinized

and washed in cold PBS. The diphenylamine assay was performed as previously described (24). Tubes containing nuclear and cytoplasmic fractions were incubated 16 to 20 hours at 30° C. Optical density was read at 600 nm.

TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE ASSAY

Cytospin preparations were made in the same manner as described above for DAPI staining. Each slide preparation received 50 μ L of TdT buffer (0.1 M sodium cacodylate (pH 7.0) (Sigma), 1 mM calcium chloride (Sigma), 0.1 mM dithiothreitol (Sigma), 0.05 mg/mL bovine serum albumin, 10 units terminal transferase (Boehringer Mannheim) and 0.5 nM fluorescein-16-dUTP (Boehringer Mannheim) (24). Cells were incubated in this solution for 1 hour at 30° C in the dark. Slides were then rinsed three times in PBS. Fluorescence was visualized using a Axiophot microscope (Zeiss).

WESTERN BLOTTING

Preparation of cellular lysates and immunoblotting are as previously described (24). Equal amounts of protein lysates (approximately 100 μ g total protein) were loaded on 6%, 10%, or 15% polyacrylamide gels and then electroblotted (Hoeffer) at 80 mA for 45 mins onto Hybond-ECL membrane (Amersham). The membrane was blocked with 20% bovine serum albumin (Sigma) in PBS overnight at RT and then incubated with a 1:100 dilution of either mouse anti-human p53 (Oncogene Science Ab-2), mouse anti-human p21/WAF1 antibody (Oncogene Science Ab-1), or mouse anti-human pRB antibody (PharMingen) for one hour at RT with agitation. The membrane was washed three to five times at RT with 250 mL PBS containing 0.1% TWEEN and then incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) at a 1:35,000 dilution for 1 at RT. The blot was washed again and complexes detected by using ECL Western Blotting Detection Reagents (Amersham) as described by the manufacturer. Detection of p27 protein was detected with a polyclonal rabbit antiserum to p27 protein at a 1:1000 dilution

(gift of Jim S. Robert), washed as described above, and detected with horseradish peroxidase-conjugated Protein A (Sigma) at a 1:2000 dilution and detected using ECL Western Blotting Detection system.

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Dysregulated bcl-2 expression inhibits apoptosis but not differentiation of retinoic acid
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Table 1: Effects of retinoic acid on HMEC cell cycle

Cells/Retinoic Acid	% G ₀ /G ₁	% change	% S	% change	% G ₂ /M
AG11132					
No Retinoic Acid	72	--	21	--	7
0.1 μ M RA	76	+6	12	-43	11
1.0 μ M RA	84	+17	9	-58	7
AG11134					
No Retinoic Acid	71	--	19	--	11
0.1 μ M RA	79	+11	11	-42	11
1.0 μ M RA	80	+13	6	-68	13

HMEC strains AG11132 (passage 14) and AG11134 (passage 15) were treated with 1.0 μ M RA for 2 days. The distribution of cells in the various phases of the cell cycle was determined by flow cytometry as described in Materials and Methods. These data are representative of three separate experiments.

Figure 1: Expression of RAR α and RAR β mRNA in RA-treated HMECs.

Northern analysis of HMEC strains AG11132 (passage 12) and AG11134 (passage 10) treated for 48 hours with (+) and without (-) 1.0 μ M RA, demonstrating the expression of the RAR α and RAR β mRNA. 10 μ g of RNA were loaded per lane. Autoradiographic exposure times are 4 and 14 days for RAR α and RAR β , respectively. 36B4 is a 1.5 kb mRNA expressed uniformly in all breast cell strains or lines and, therefore, serves as a loading control (64).

Figure 2: RA-mediated *trans*-activation of β RARE in HMECs.

CAT reporter assays of RA-treated HMEC strains AG11132 (passage 11 to 14) (A) and AG11134 (passage 9 to 12) (B) transfected with pRRE4-tkCAT plasmid. Cells were treated with 0, 0.1, 1.0, and 10 μ M RA for 24 hours. pCMV-GH was used as a transfection control. CAT counts were corrected for growth hormone activity and total protein (see Materials and Methods). Data represent the mean of three independent transfections performed in duplicate.

Figure 3: RA-mediated growth inhibition of HMECs.

Growth curves of HMEC strains AG11132 (passage 12) (A) and AG11134 (passage 10) (B). Cells were plated on Day -1 in standard medium in duplicate at 1×10^4 cells per well. Cells were re-fed on Day 0 with standard medium containing 0, 0.1, or 1.0 μM RA. Untreated controls received an equivalent volume of ethanol (0.1%). Cells were trypsinized at 12 to 24 hour time intervals and counted in triplicate.

Figure 4: Effect of RA on cell cycle phase distribution in HMECs.

Cell cycle distribution of HMEC strains AG11132 (passage 14) (A) and AG11134 (passage 14) (B) treated with 1 μ M RA. Cells were plated on Day -1 in standard medium, re-fed on day 0, and treated with 1 mM RA on days 0, 1, 2, and 3. Cells were harvested on day 4. (See Materials and Methods.) Data are presented relative to the %S and %G₁ phase of the untreated cells and are representative of 3 separate experiments.

Figure 5: Nuclear staining of RA-treated HMECs.

HMEC strains AG11132 (passage 13) (A,C) and AG11134 (passage 11) (B,D) were incubated with (C,D) and without (A,B) 1.0 μ M RA acid for 5 days and then stained with DAPI as described in Materials and Methods. HMECs treated with RA did not exhibit nuclear condensation characteristic of apoptosis.

Figure 6: Cytoplasmic DNA fraction in RA treated HMECs.

The indicated cells were treated for 0, 2, 4, and 6 days with 1.0 μ M RA. Cytoplasmic DNA was determined by the diphenylamine assay as described in Materials and Methods. HMEC strains AG11132 (passage 12) and AG11134 (passage 10) do not demonstrate cytoplasmic fragmented DNA. HL-60 cells exhibit retinoic acid-induced apoptosis (68) and serve as a positive control.

Figure 7: Expression of c-myc and p27 mRNA in RA-treated HMECs.

Northern analysis of HMEC strains AG11132 (passage 12) and AG11134 (passage 10) treated for 48 hours with (+) and without (-) 1.0 μ M RA, demonstrating the expression of the c-myc and p27 mRNA. 10 μ g of RNA were loaded per lane. Exposure time was 3 days and 7 days respectively for c-myc and p27. 36B4 is a 1.5 kb mRNA expressed uniformly in all breast cell lines and therefore serves as a loading control (64).

Figure 8: Expression of pRB, p53, and p21 protein following treatment with all-*trans*-retinoic acid.

AG11132 (passage 11) and AG11134 (passage 12) treated for 48 hours with (+) and without (-) 1 μ M RA and analyzed for pRB, p53, and p21 protein expression. Equal amounts of protein lysate were loaded per lane. Incubation was with a 1:100 dilution of anti-pRB antibody (PharMingen), a 1:100 dilution p53 specific antibody (Oncogene Science), or a 1:100 dilution of p21 specific antibody (Oncogene Science) and detected by chemiluminescence as described in Materials and Methods. The arrow denotes the location of the hyperphosphorylated pRB protein. The protein gel was stained with Coomassie blue and an unidentified 45 kd protein was used as a loading control.

Figure 9: Relative expression of hyperphosphorylated pRB following treatment with all-*trans*-retinoic acid.

(A) AG11132 (passage 10) and (B) AG11134 (passage 8) were treated for 0, 24, and 48 hours with 1 μ M RA and analyzed for relative levels of hyperphosphorylated and phosphorylated pRB protein expression. Equal amounts of protein lysate were loaded per lane. Levels of pRB were detected utilizing a 1:100 dilution of antibody to pRB (PharMingen) and detected with chemiluminescence (Amersham). Densitometer readings were made from resulting autoradiograph. Readings were standardized relative to an unidentified 45 kd protein detected by Coomassie blue staining of the original gel.

Figure 1

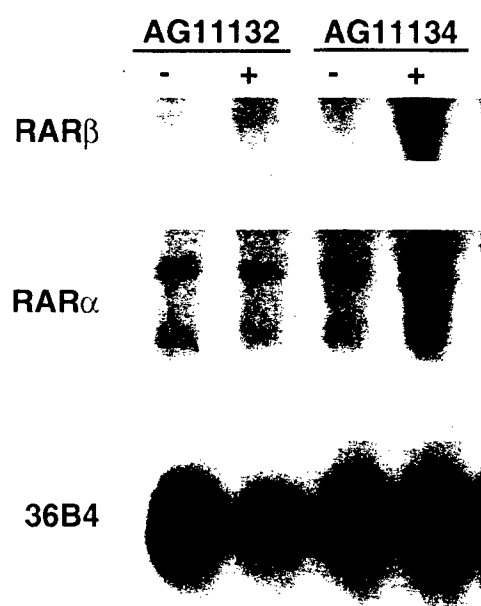


Figure 2

A AG11132

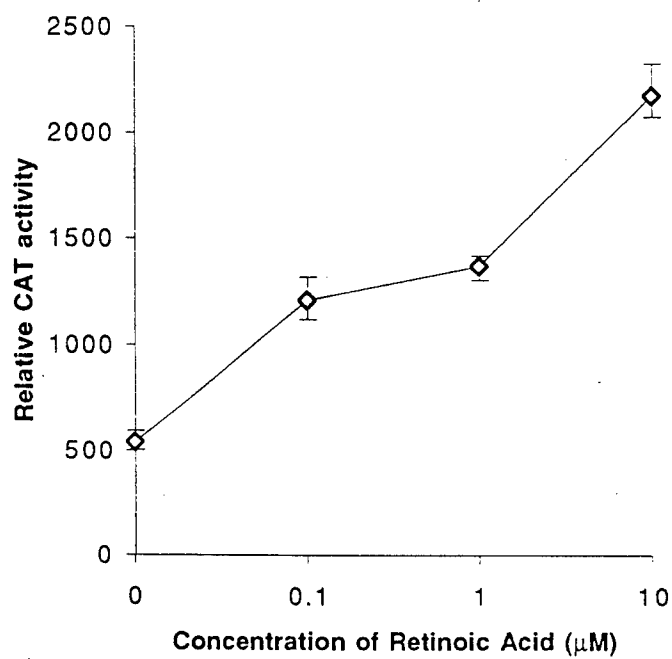


Figure 2

B AG11134

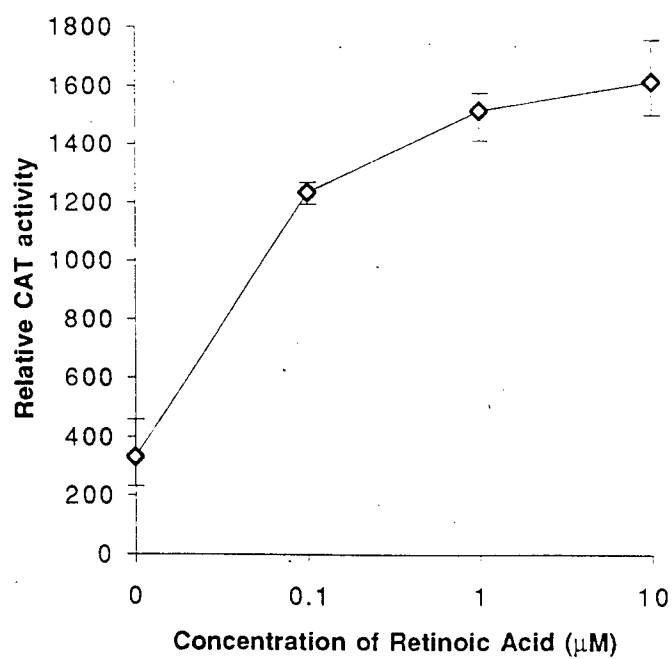


Figure 3

A AG11132

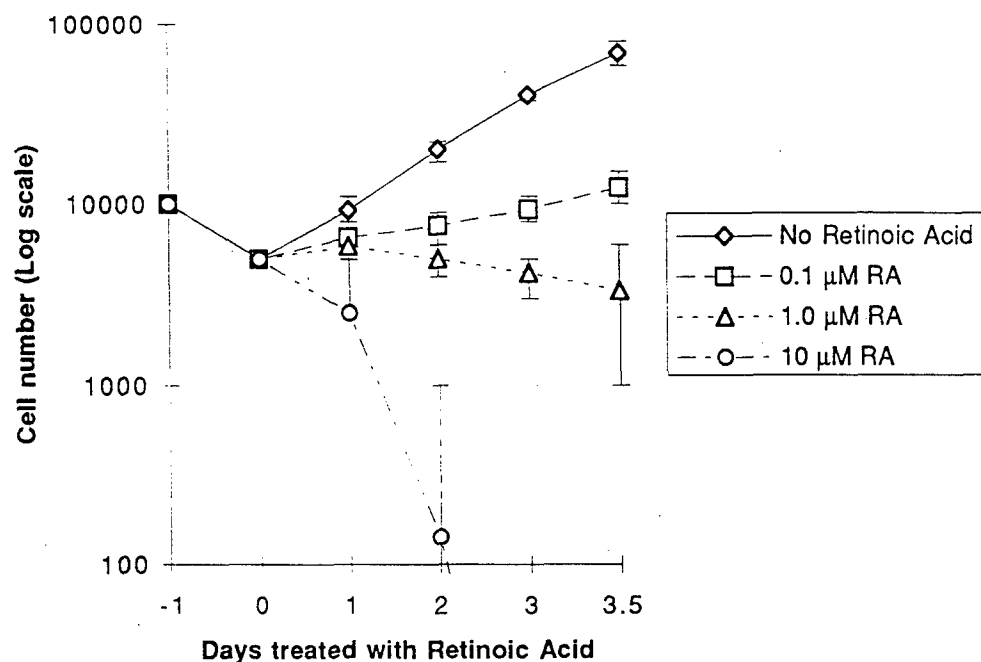


Figure 3

B AG11134

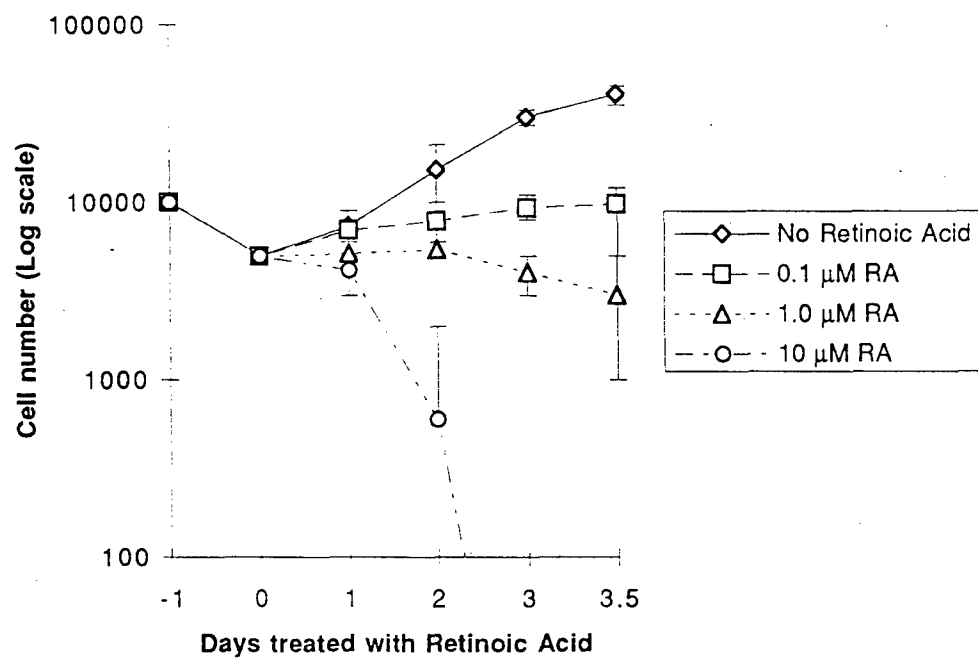


Figure 4

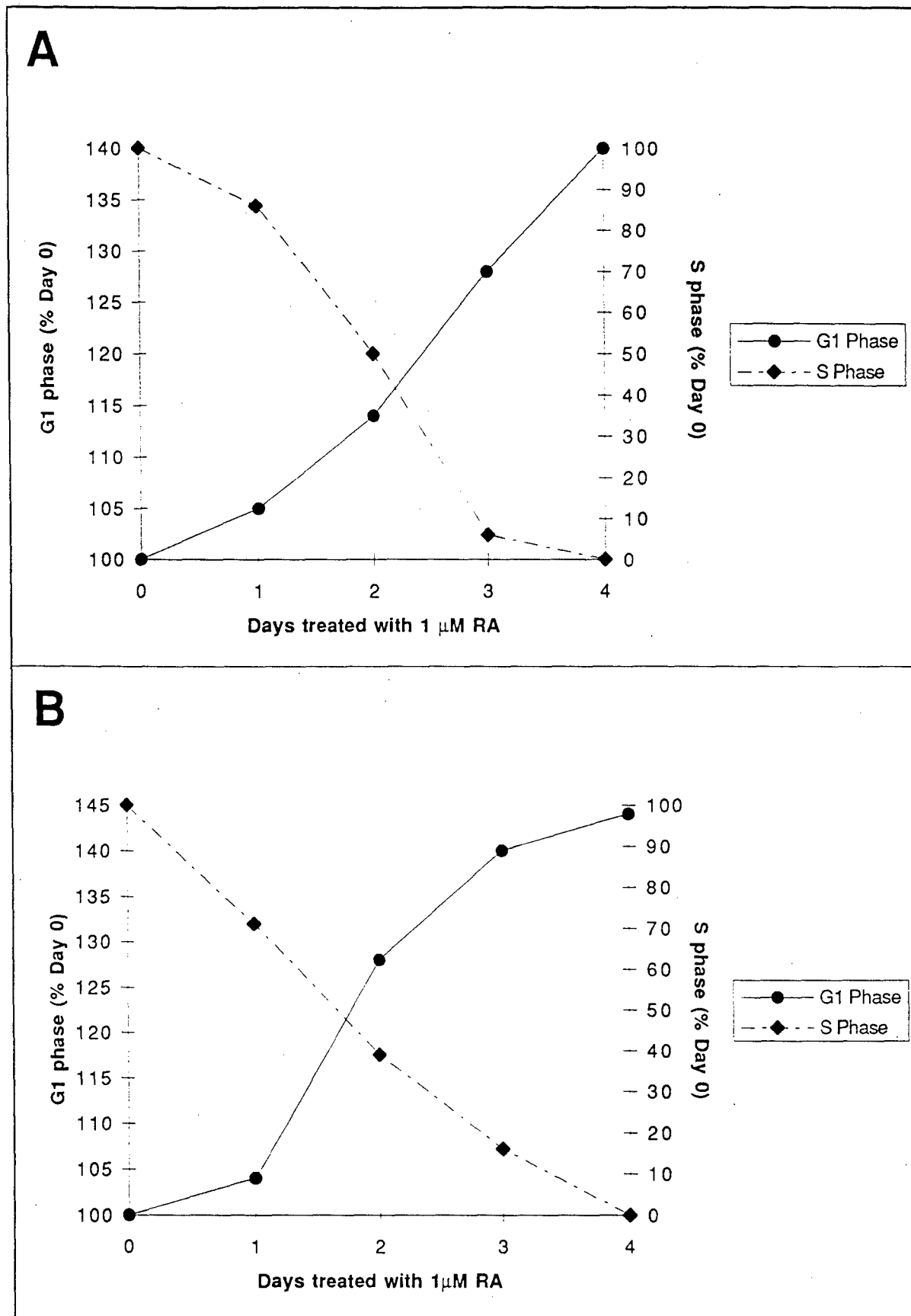


Figure 5

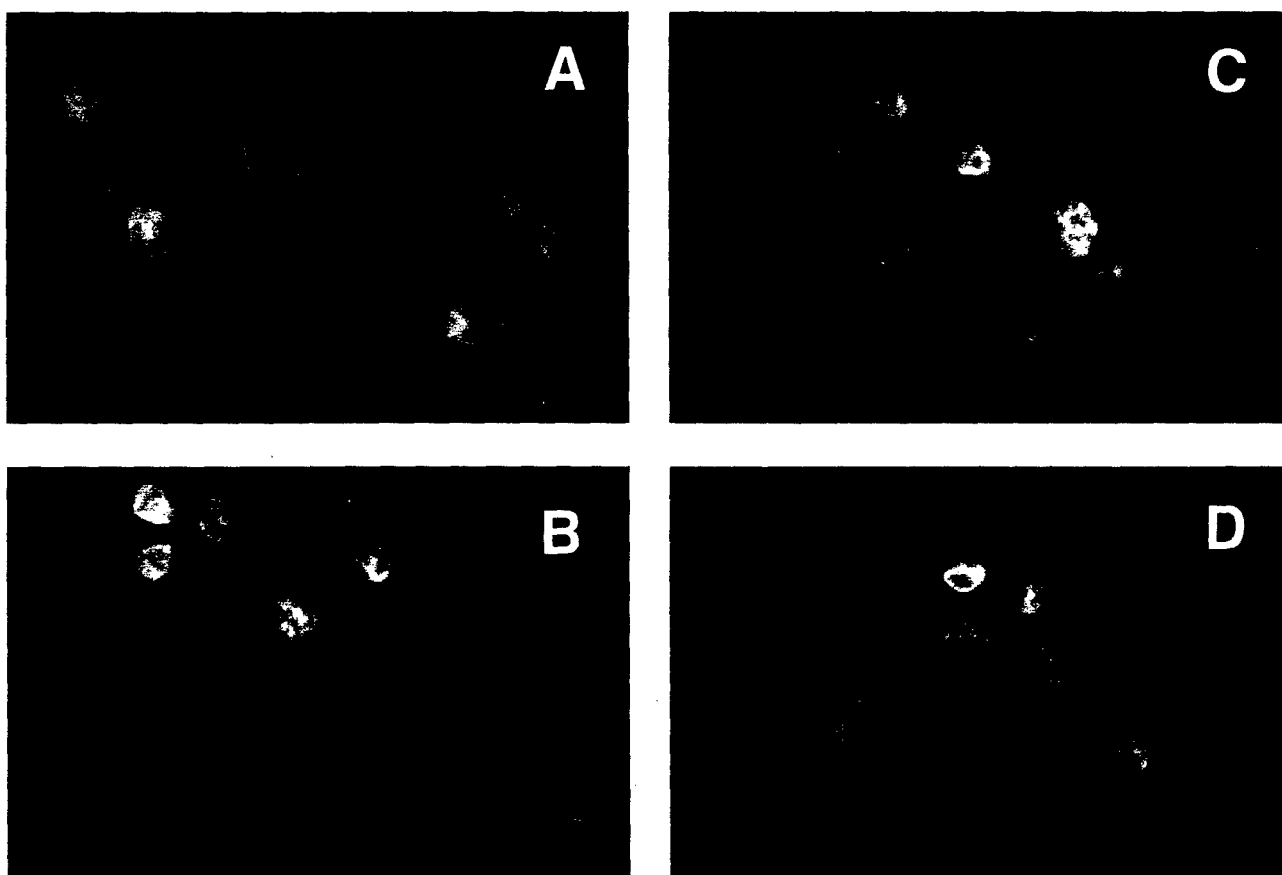


Figure 6

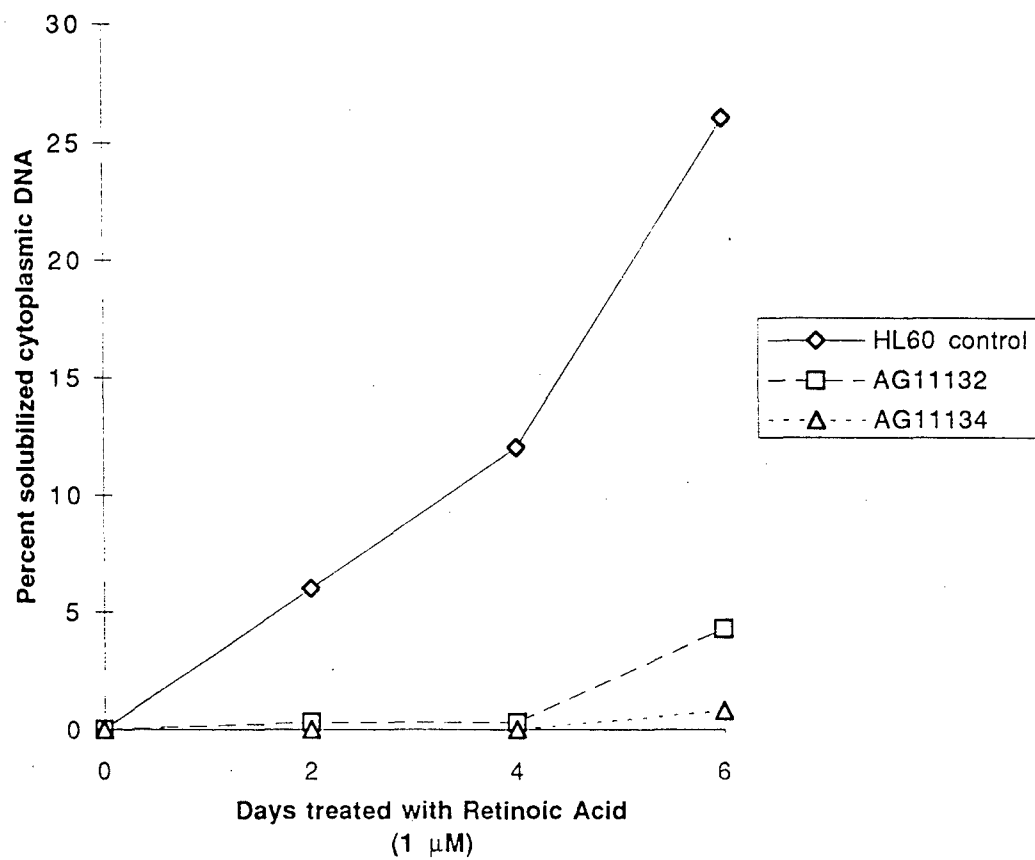


Figure 7

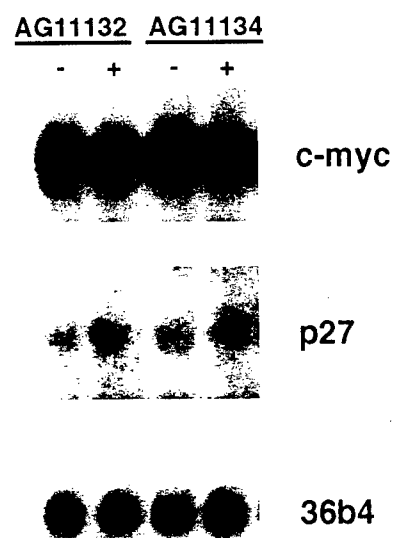


Figure 8

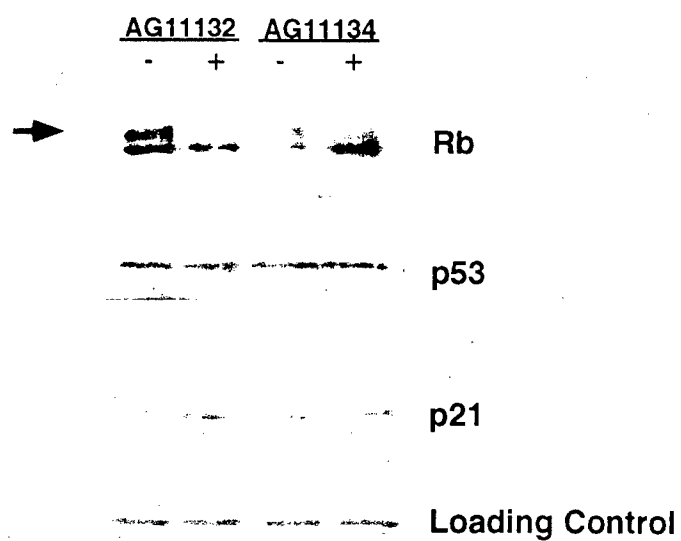


Figure 9

A

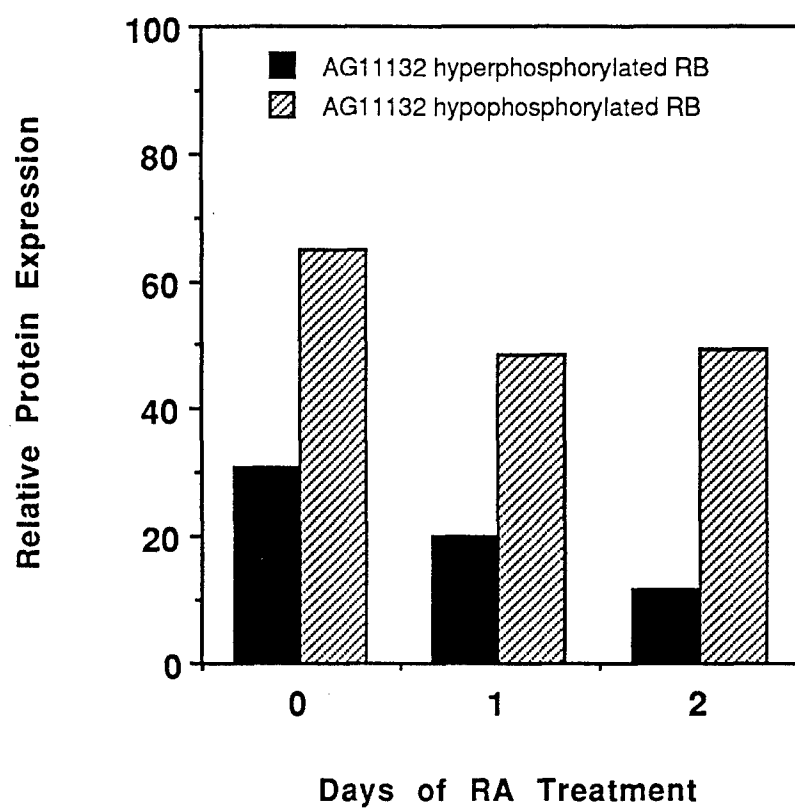
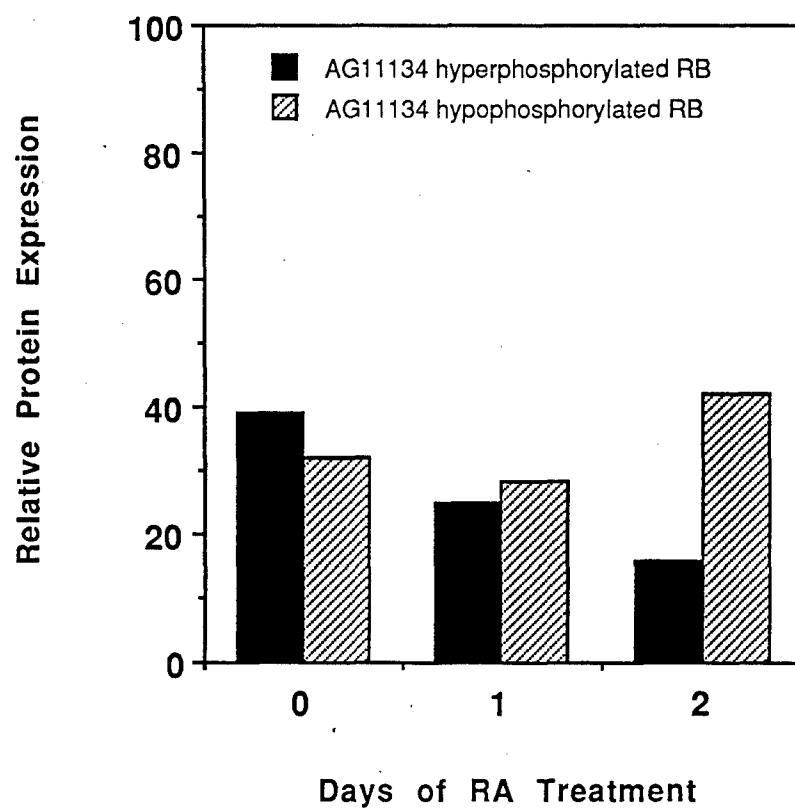


Figure 9

B





DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

6 May 98

MEMORANDUM FOR Administrator, Defense Technical Information
Center, ATTN: DTIC-OCF, Fort Belvoir,
VA 22060-6218

SUBJECT: Request Change in Distribution Statement

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2. Point of contact for this request is Ms. Betty Nelson at DSN 343-7328 or email: betty_nelson@ftdetrck-ccmail.army.mil.

FOR THE COMMANDER:

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management