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RETINOIC ACID NUCLEAR RECEPTOR α (RARα), A MAJOR ROLE IN MEDIATING RETINOIDS INHIBITION OF GROWTH IN HUMAN BREAST CARCINOMA CELLS

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Retinoids mediate their actions via RARs (retinoic acid receptors) and RXRs (retinoid X receptors). Each classes of these nuclear retinoid receptor is further subdivided into three species namely α, β and γ. Recent studies demonstrate that ER - positive HBC cell lines are sensitive and ER - negative cell lines are resistant to growth inhibitory effects of retinoic acid (RA). In this study we look at the expresion of RARs and RXRs in 6 HBC cell lines, we found only RARα mRNA level was strong correlated with ER - status. To further investigate the major role of RARα in retinoid - mediated inhibition of growth, we transfected RARα cDNA in two RA - resistant ER - negative HBC cell lines. Analyses of different clonal populations of RARα transfectants from each cell line revealed growth inhibition by retinoids. Our results demonstrates that RARα plays a major role in mediating retinoids inhibition of growth in HBC cells and adequate levels are required for such actions.

Key words: Retinoids, Breast cancer, Nuclear receptors, Stable transfection.

A number of studies have now shown that retinoids, the natural and synthetic derivatives of vitamin A, are highly effective in preventing the development of mammary carcinoma in a number of systems.[1] Retinoid acid (RA) has also been shown to play an important role in the differentiation of a number of cell types of endodermal, ectodermal, and mesodermal origin.[2] Retinoids act by binding to specific nuclear receptor that belong to the steroid/thyroid hormone nuclear receptor superfamily.[3] So far two classes of retinoid receptor, the RARs and RXRs, have been identified. Each classes is further subdivided into three distinct type namely RARα, β, γ, and RXRα, β, γ.[4] The ligand activated RARs and RXRs form either homodimers or heterodimas and function as trans - acting nuclear transcription factors that bind to their respective response elements knows as RAREs.[4] There are some data showing that a correlation exits between RA inhibition of human breast carcinoma (HBC) cell growth and the estrogen receptors (ER) status.[5] RA selectively inhibited the growth of the ER - positive HBC cells; ER - negative HBC cells were refractory to the inhibition effects of RA.[5] The mechanism by which retinoids inhibit breast carcinoma growth remains unclear. In this study we try to find the potential mechanism of how RA

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inhibits HBC cell growth.

MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagles's Medium, Ham's F - 12 medium and fetal bovine serum were obtained from Gibco - BRL (Grand Island, NY). Sulfatase, dextran, charcoal, HEPES and G418 were purchased from Sigma Chemicals (St. Louis, MO). [^{32}P] dCPT (3000 Ci/mmol) was purchased from Amersham (Arlington Hts. IL).

Cell Line and Cell Cultrue: All the human breast carcinoma cell lines were routinely cultured in Dulbecco's Modified Eagle: Ham's F - 12 (1:1) medium supplemented with FBS as described in Fontana et al:[6]

Retinoids

All - trans - retinoid acid was purchased from Sigma Chemicals (St. Louis, MO). [^{3} H] RA (55.7 Ci/mmol) was purchased from DuPont - New England Nuclear (Boston, MA).

Growth Experiments

For growth experiments cells were plated in Dulbecco's Modified Eagle: Ham's F - 12 (1:) medium supplemented with 5% FBS for a period of 24 hours. The cells were then treated with RA for three, six and nine days in the same medium; control cells were treated with vehicle alone and the medium and the RA was changed every two days. Subsequently, the cells were trypsinized and counted using a hemocytometer.

Stable Transfections

For stable transfection of RARα the MDA - MB - 231 and MDA - MB - 468 cells (both are ER - negative cells) were initially plated at a density of 1×10^5 cells/100 - mm dish 24 hours before transfection in regular medium. 10 μg of the expression plasmid pSG5 - RARα and 2 μg of the dominant selection vector pSV2neo were cotrans - fected using the calcium phosphate - DNA coprecipitation method as described in Sambrook et al:[7] Control cells weere transfected with the pSV2neo vector alone (mock - transfected). G418 - resistant clones were selected in medium containing 600 μg G418/ml and were expanded. The RARα - positive colonies were identified by Northern blot analysis and RA - binding study.

Northern Blot Analysis and cDNA Probes

RNA extractionn and Northern blot analysis were essentially as described in Sheikd et al:[8] The full length human RARα cDNA was excised from the expression plasmid pSG5 - RARα with *Eco*RI and gel purified. Probe labeling was according to the random primer method of Feinberg and Vogel - stein.[9]

Assay of RA - binding Activity

The logrithmically growing RARα trans - fectants and the mock transfectants were harvested and washed several times with 1X PBS; nuclear extracts were prepared as desscribed in Jetten et al:[10] For RA - binding activity the nuclear extracts were incubated with 5 nM [^{3} H] RA (55.7 Ci/mmol) in the presence and absence of a 200 - fold excess of unlabeled RA and analyzed on Sepharose 12 HR 10/30 colomn as previously described.[10] The radioactivity was determined with a radioflow detector using the software provided by the manufacturer.

RESULTS

RARs and RXRs mRNA Expression in HBC Cells

To investigate how RARs and RXRs expres - sion in HBC cells, we looked at RARs and RXRs mRNA levels expression. We found that all the ER

– positive cell lines tested exhibited higher levels of RARα mRNA (Figure 1). Very low levels of RARβ and RXRγ mRNA were detected and no statistically significant correlation with ER – status was found (data not show). RARγ and RXRα mRNA expression were comparable in all the cell lines regardless of the ER – status (Figure 2). We could not adequately assess RXRβ mRNA levels because the strong cross hybridization of RXRβ cDNA probe with 18S and 28S RNAs.

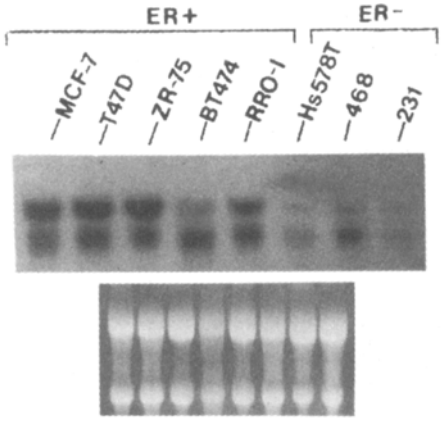

Fig. 1. RARα mRNA levels in ER positive and negative human breast carcinoma cell lines. The cell lines were grown, total RNA extracted and hybridizations performed as described in materials and methods. MCF – 7, T – 47D, ZR – 75 and BT – 474 ER – positive; Hs578, MDA – MB – 231 and MDA – MB – 468 ER – negative. Ethidium bromide staining of the gel revealed RAN integrity and comparable loading in each lane.

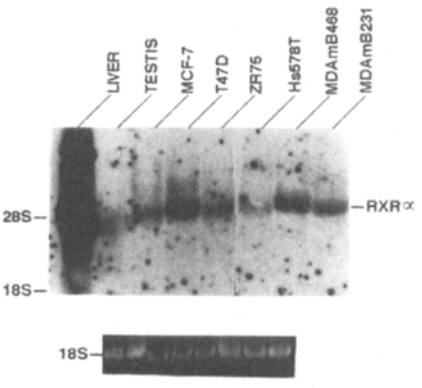

Fig. 2. RXRα mRNA levels in ER positive and negative human breast carcinoma cell lines. The cell lines were grown, total RNA extracted and hybridizations performed as described in materials and methods. MCF – 7, T – 47D and ZR – 75 ER – positive; Hs578, MDA – MB – 231 and MDA – MB – 468 ER – negative. Ethidium bromide staining of the gel revealed RAN integrity and comparable loading in each lane.

⟧ Stable Transfection of RARα cDNA in ER – negative HBC Cells

In all the the RARs and RXRs expression, we found that only RARα mRNA exhibited higher levels in ER – positive cell lines which these cells exhibited sensitivity to growth inhibition by RA. This results let us to postulate that RARα is the major mediator of retinoids action in HBC cells and adequate levels of RARα are required for retinoid inhibition of HBC growth. To conform these hypothesis, the two retinoid – resistant ER – negative transfected with expression vectors carrying RARα cDNA under control of a SV40 promoter. Several G418 resistant clones were tested for the integration of the transfectants. Northern blot analysis revealed differential expression of transfected RARα cDNA recombinant mRNA (Figure 3). Two RARα transfectants clone 7 from MDA – MB – 231 and clone 27 from MDA – MB – 468, expressing higher levels of recombinant RARα mRNA were further characterized by HPLC for RA – specific binding. The results show that RARα transfectant MDA – MB231 and MDA – MB – 468 cells exhibit an approximately 4 – fold higher [^{3}H] RA – binding than their respective mock – tansfectant counterpart (Table1).

RA Inhibited the Growth of RARα – transfected

ER – negative HBC Cells

We next investigated the effects of RA treatment on the proliferation of the RARα – transfectants. Clones 7 and 27 expressed higher levels of RARα and clones 4 and 19 expressed low levels of RARα representing RARα – transfected MDA – MB – 231 and MDA – MB – 468 HBC cells respectively, were treated with RA. As demons – trated in Figure 4, 1 μM RA inhibited the growth of higher expression clones while no effect on the proliferation of low expression clones and mock – transfected counterparts. The data shown in Table 2 demonstrate that RA inhibited the growth of RARα transfectants in dose dependent of approximately 1 μM in both higher expression cell.

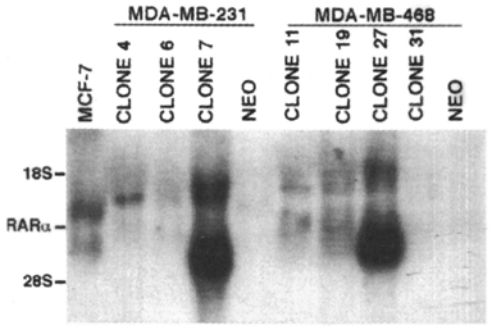

Fig. 3. Northern blot analysis of total RNA from various G418 – resistant clones of RARα – transfected and pSV2neo – transfected MDA – MB – 231 and MDA – MB – 468 HBC cells. Total cellular RNA (25 μg) per sample was analyzed and the blot was probed with human RARα cDNA probe. RNA from ER – positive MCF – 7 cells was included as the positive control.

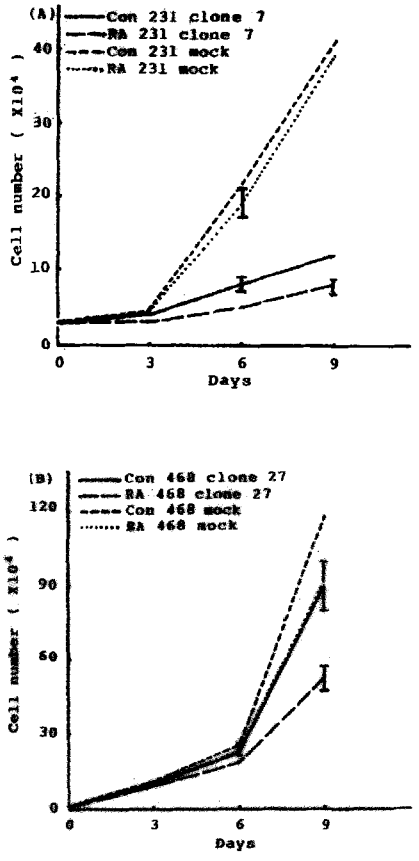

Fig. 4. Effect of RA on the growth of mock – transfected and RARα – transfected MDA – MB – 231 (A) and MDA – MB – 468 (B) HBC cells. Cells were seeded in DMEM/F12 supplemented with 5% FBS for 24 h and treated with 1 μM RA as described in materials and methods. The results represent of two – independent exprements.

DISCUSSION

In this study, we provided direct evidences that the expression of adequate RARα is associated with the ability of RA to inhibit HBC cells proliferation. Although the mechanism of RA inhibits HBC growth remains to be firmly esta – blished, we have presented several lines of evidence that adequate levels of RARα are critical for RA action in HBC cells and are responsible for RA ability to inhibits HBC proliferation. First, the ER – negative cells are not growth inhibited by RA and they inherently express lower levels of RARα, the ER – positive cells are growth inhibited by RA and

Table 1. [^3H]RA – binding to nuclear extracts from mock – transfected and RARα – transfected MDA – MB – 231 and MDA – MB – 468 cells

Cell line	[^{3}H]RA – binding (dpm/10^7 cells × 10^{-3})
MDA – MB – 231 (mock – transfected)	1.1
MDA – MB – 231 (clone 7)	4.3
MDA – MB – 468 (mock – transfected)	1.5
MDA – MB – 468 (clone 27)	7.3

Preparation of nuclear extracts and analysis of [^{3}H]RA – binding was as described in materials and methods. The values represent means of two – independent determinations and deviation between the values did not exceed 10%.

Table 2. Dose dependent growth inhibition of RARα transfectants by RA.

Retinoid Conc.	MDA – MB – 231(clone 7)	MDA – MB – 468(clone 27)
	% Inhibition (day 9)	% Inhibition (day 9)
Experiment 1		
10nM	5.8 ± 2.3	4.5 ± 1.0
0.1 μM	25.3 ± 5.8	31.9 ± 4
0.5 μM	43.3 ± 2.3	50.3 ± 2
1.0 μM	48.8 ± 1.1	56.1 ± 2.3
10 μM	57.1 ± 2.8	61.8 ± 2.9
Experiment 2		
10nM	7.0 ± 4.6	5.8 ± 6.3
0.1 μM	20.3 ± 4.6	25.3 ± 1.9
0.5 μM	43.3 ± 2.3	43.3 ± 1.2
1.0 μM	48.4 ± 3.4	48.8 ± 3.1
10 μM	57.1 ± 8.6	54.1 ± 1.2

Cells (2×10^4) were seeded in duplicate wells as described in material and methods. RA treatment was for 9 days with fresh RA and medium change every 2 days.

they express much higher levels of RARα than ER – negative cells. Second, the express of exoge – nously transfected RARα at higher levels in different retinoid resistant ER – negative cells resulted in acquisition of ssensitivity to growth inhibition by RA, and low levels of RARα and non – transfected or mock – transfected controls did not show any inhibition. Because the retinoids in this study did not show any inhibitory effects in mock – transfeected or non – transfected parental cells, the growth inhibitory effects of retinoids must be due to the presence of trransfected RARα.

ER – status also plays a important role in retinoids inhibition.[5] Retinoids selectively inhibited the growth only in ER – positive HBC cells, and ER – negative cells were refractory to the inhibition effects of RA.[5] Sheikh et al. demonstrated that the ER – transfected ER – negative MDA – MB – 231 cells made these cells sensitively RA – mediated growth inhibition.[11] In these cells not only constitutively express higher levels of RARα mRNA than their parental counterparts, but also exhibit higher RA – mediated CAT activity from RARE/tk – CAT constructs than the ER – negative parental

cells. Also estrogen can up - regulate the expression of RARα both ER - transfected cells and well established ER - positive HBC cell lines and this up - regulate RARα levels was associated with acquisition of sensitivity RA inhibition of growth.

In conclusion, we have demonstrated that RARα plays a major role in mediating retinoids inhibition of growth in HBC cells, and adequate levels are required for such action. In clinic RARα levels may serve as a potential marker to determine patients responsiveness to retinoids therapy.

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