In Vitro Inhibition of Proliferation of Estrogen-Dependent and Estrogen-Independent Human Breast Cancer Cells Treated with Carotenoids or Retinoids

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ABSTRACT Both estrogen-receptor (ER) positive MCF-7 and ER-negative Hs578T and MDA-MB-231 human breast cancer cells were treated with carotenoids (β-carotene, canthaxanthin and lycopene) and retinoids (all-trans-, 9-cis- and 13-cis-retinoic acid and all-trans-retinol). Among carotenoids, β-carotene significantly reduced the growth of MCF-7 and Hs578T cells, and lycopene inhibited the growth of MCF-7 and MDA-MB-231 cells. Canthaxanthin did not affect the proliferation of any of the three cell lines. All-trans- and 9-cis-retinoic acid significantly reduced the growth of both MCF-7 and Hs578T cells, whereas 13-cis-retinoic acid and all-trans-retinol had a significant effect only on MCF-7 cells. MCF-7 and Hs578T cells treated with all-trans-retinoic acid (all-RA) were further studied for the mechanism behind growth inhibition. Retinoic acid receptors α and γ (RARα, γ) in MCF-7 cells and RARα, β and γ in Hs578T cells were not induced by all-RA treatment at either the protein or mRNA level. Hs578T cells treated with all-RA had significantly more cells in the G0/G1 stage of the cell cycle, but the same was not observed for MCF-7 cells. All-RA induced a dose-dependent cell death in MCF-7 cells, which may be a necrotic phenomenon. These results demonstrate that ER status is an important, although not essential factor for breast cancer cell response to carotenoid and retinoid treatments, and the mode of action of all-RA in MCF-7 and Hs578T cells is not through the induction of RAR. Other mechanistic pathways that are either followed by or concomitant with growth inhibition are possible. J. Nutr. 131: 1574–1580, 2001.

KEY WORDS: β-carotene • retinoic acid • breast cancer cells • cell cycle • retinoic acid receptors

Cancer of the breast is the most common incident cancer and cause of death from cancer in women (WHO 1997). The offspring of emigrants from countries with low breast cancer incidence to countries with high breast cancer incidence acquire rates close to those of the new country, suggesting that environmental and lifestyle influences are important in the etiology of breast cancer (McMichael and Giles 1988). This could be the result of noninherited factors including, among other possibilities, diet (Trichopoulos and Willett 1996).

Epidemiologic and laboratory investigations exploring the relationship between diet and disease suggest an inverse correlation between consumption of fruits and vegetables rich in carotenoids and certain cancer incidence rates (Block et al. 1992, Ziegler 1989). In a review of possible environmental determinants of cancer, it was estimated that 35% of cancers in the United States might be attributable to dietary factors (Doll and Peto 1981). Several studies have shown that low levels of either dietary intake or plasma carotenoids are associated with an increased risk of breast cancer (Comstock et al. 1992, Potischman et al. 1990, Weisburger 1991).

Carotenoids may act as chemoprotective agents through biological activities such as metabolism to retinoids, antioxidation, immunomodulation, protection against cellular mutagenesis and malignant transformation, and inhibition of tumorigenesis (Bendich 1989, Krinsky 1989 and 1992, Shults et al. 1992). Carotenoids also function as radical-trapping antioxidants in vitro and may have the same activity in vivo (Burton and Ingold 1984, Krinsky 1989). Retinoids, on the other hand, exert a variety of effects on basic biological processes such as growth, differentiation, development and malignant transformation, in addition to receptor-induced signal transduction. Knowledge of the effect of these compounds has led to the hypothesis that retinoids may act as chemopreventive agents as well as inhibitors of the growth of established tumors (Hill and Grubb 1992, Peto et al. 1981).

This study was designed to determine the effects of carotenoids and retinoids on the growth of human breast cancer cells. Briefly, the growth of estrogen-receptor positive (ER+) MCF-7 and estrogen-receptor negative (ER- Hs578T and MDA-MB-231 human breast cancer cells was determined after

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treatment with a provitamin A carotenoid, β-carotene, and the nonprovitamin A carotenoids, canthaxanthin and lycopene, and the retinoids, all-trans-, 9-cis-, and 13-cis-retinoid acid and all-trans-retinol. The mechanism underlying growth inhibition caused by all-trans-retinoid acid (all-\( \text{t-RA} \)) in MCF-7 and HS578T cells was further studied by determining the retinoid acid receptor (RAR) expression, cell cycle arrest and apoptosis induction in these cells.

### MATERIALS AND METHODS

#### Chemicals.
Growth media and other growth regulators were purchased from Gibco Life Technologies (Gaithersburg, MD). Crystalline β-carotene, canthaxanthin, all-trans-, 9-cis-, and 13-cis-retinoid acid and all-trans-retinol were purchased from Sigma Chemical (St. Louis, MO). Crystalline lycopene was kindly provided by LycoRed, Beer-Shiva, Israel. Tetrahydrofuran (THF) stabilized with BHT and acetone was purchased from Fisher Scientific, Pittsburgh, PA. Crystalline RNA probes were obtained as a kind gift from the laboratory of Dr. Monica Peacocke, Columbia University, New York, NY.

#### Cells and cell culture conditions.
MCF-7 human breast cancer cells were obtained from the Michigan Cancer Foundation (Detroit, MI). HS578T and MDA-MB-231 human breast cancer cells were obtained from the American Type Culture Collection (Rockville, MD). MCF-7 cells were maintained in α-MEM medium containing HEPES, MEM nonessential amino acids, sodium pyruvate, L-glutamine, insulin, gentamycin and 10% fetal bovine serum (5% serum for the experiments). HS578T cells were maintained in Dulbecco’s modified Eagle’s medium with 4.5 g/L glucose, 10 mg/L insulin and 10% fetal bovine serum. MDA-MB-231 cells were maintained in a 1:1 mixture of Dulbecco’s modified Eagle’s medium with Ham’s F-12 medium containing 2 mmol/L L-glutamine, and 10% fetal bovine serum. Cells were grown in 100-mm culture dishes and incubated at 37°C in a humidified atmosphere of 5% CO2 in air. Cells were seeded at a density of 5000 cells/cm² for the experiments, unless otherwise indicated. The trypan blue exclusion method using a hemocytometer was used to distinguish viable from dead cells.

#### Preparation of carotenoid and retinoid solutions.
Solutions of β-carotene, canthaxanthin and lycopene were prepared in THF containing 0.25 g/L BHT as a preservative. Fresh solutions of the carotenoids were prepared in a nitrogen environment in a plastic glove box containing 0.25 g/L BHT as a preservative. Fresh solutions of the carotenoids were prepared to result in concentrations of 1, 3, 7, 10 and 20 mmol/L. The appropriate stock solution (1 mmol/L) was subjected to electrophoresis on a 1.2% agarose gel and the purity of the carotenoid and retinoid acid and all-trans-retinol was determined in dimethyl sulfoxide to produce stock solutions of 0.1 mmol/L. Further dilutions were made in acetone. Retinoids were then dissolved in the growth media to reach final concentrations of 10 nmol/L, 100 nmol/L and 1 µmol/L at a 0.25% concentration of acetone that was toxic to the cells (data not shown). The purity of the carotenoid and retinoid stocks was determined by HPLC and the concentration was determined by spectrophotometric analysis. Red light was used during preparation of carotenoid and retinoid solutions to prevent photodegradation of these compounds.

#### Treatment of cells.
Treatments were applied 24 h after cultures had been seeded to ensure proper attachment of the cells to the plastic culture plates or dishes. MCF-7, HS578T and MDA-MB-231 cells were incubated with different carotenoids or retinoids for the periods mentioned in the respective experiments. Control cells received medium supplemented with THF or acetone only. Control and treatment media were changed every other day.

#### Cell proliferation.
For the determination of cell proliferation, MCF-7, HS578T and MDA-MB-231 cells were incubated with β-carotene, canthaxanthin, lycopene, all-trans-, 9-cis- and 13-cis-retinoid acid and all-trans-retinol solutions for up to 9 d. Proliferating viable cells attached to the plastic wells were harvested and the numbers counted on d 2, 5 and 9 (data are shown for d 9 only, i.e., when the cells reached confluency). For cell harvesting, the medium was aspirated from the wells; 0.5 mL of 0.25% trypsin solution was added to each well and incubated at 37°C for 5, 3 and 2 min, for MCF-7, MDA-MB-231 and HS578T cells, respectively. The reaction was stopped with 1.5 mL of the growth medium at room temperature. The cells were collected and counted in duplicate using an electronic Coulter Counter (model Z1; Coulter, Miami, FL). Cell morphology was monitored by periodic evaluation of the cells under a phase contrast microscope throughout the experiments.

#### Northern blot analysis of RAR(α, β and γ) mRNA expression.
MCF-7 and HS578T cells were incubated with all-t-RA for 2, 6, 8, 24 and 72 h. RARα, β and γ expression was examined at the transcription level in the control and treatment cells by Northern blot analysis of total cellular RNA, which was extracted from the cells using the cesium chloride gradient method (Tsou et al. 1994). RNA from each sample (30 µg) was subjected to electrophoresis on a 1.2% agarose gel containing 5% formaldehyde. The gel was transferred to a Nytran Plus nylon membrane using a TurboBlotter system (Schleicher & Schuell, Keene, NH). The membrane(s) were subjected to UV-crosslinking and then hybridized to [α-32P]-dCTP-labeled retinoid receptor cDNA probes (Tsou et al. 1994). pHE7 was used as a control gene to monitor equivalent loading of RNA in each lane because the levels of pHE7 are not affected by retinoic acid (Tsou et al. 1994). After high stringency washing (0.1X standard saline citrate at 60°C), the membranes were exposed to photographic film using an intensifying screen at -80°C for various lengths of time (between 1 and 7 d). The films were developed using an RO II film processor (Fuji, Elmsford, NY).

#### Flow cytometry.
MCF-7 and HS578T cells were incubated with all-t-RA for 6 and 9 d. After exposure to experimental conditions, cells were collected and washed twice with cold PBS and suspended again in PBS. Absolute ethanol was added dropwise with vortexing at low speed to bring the ethanol concentration to 70%; this suspension was fixed at 4°C for 30 min. Cells were again washed twice with PBS after removal of ethanol and incubated with 10 mg/L RNase solution in PBS at 37°C for 30 min, with mixing several times during incubation. After being washed twice with PBS, cells were suspended in propidium iodide solution at 1 × 10⁶ cells/0.5 mL concentration. Cells were then analyzed for their cell cycle distribution using a Flow cytometer (Beckton Dickinson, Franklin Lakes, NJ) and ModFit LT 2.0 software (Beckton Dickinson) for data analysis.

#### Morphological analysis.
MCF-7 cells were incubated with all-t-RA for 7 d. Cells were grown on chamber slides for the morphologic analysis. After exposure to experimental conditions, cells were washed with PBS and stained with acridine orange (25 mg/L) and propidium iodide (25 mg/L) for 15 min in the dark. Cells were then washed in PBS, fixed in 10% formalin for 30 min in the dark and washed again in PBS. Dual-stained cells were viewed using a fluorescence microscope. Cells were coded as either early apoptotic (bright green, highly condensed chromatin), late apoptotic (bright orange, highly condensed chromatin) or necrotic cells (bright orange nucleus without condensed chromatin) (Broadus et al. 1996).

#### Statistics.
Results are expressed as means ± SD of three determinations. Comparisons of mean values of control and treatment cells were made using ANOVA on the whole population followed by two-sample Student’s t test between control and each treatment group. The statistical significance of difference (P < 0.05) for the
treatment groups was determined relative to their respective control group.

RESULTS

Effect of carotenoids on cell growth. The effects of β-carotene at 1, 3 and 10 μmol/L and lycopene at 1, 3 and 10 μmol/L, dissolved in THF (0.1%), on the growth of MCF-7, Hs578T and MDA-MB-231 human breast cancer cells grown at a density of 5000 cells/cm². Cells were treated with β-carotene at concentrations of 1, 3 and 10 μmol/L and lycopene at concentrations of 7, 10 and 20 μmol/L in the media for 9 d. Values are means ± SD of three replicate assays; *significantly different from control, P < 0.05.

Effect of retinoids on cell growth. The effects of all-trans-, 9-cis- and 13-cis-retinoic acid, dissolved in acetone (0.25%), on the growth of Hs578T cells are shown in Figure 2. All-trans- and 9-cis-retinoic acid significantly inhibited the growth of Hs578T cells at 100 nmol/L and 1 μmol/L on d 9 (P < 0.05; Fig. 1B). Canthaxanthin, at concentrations up to 20 μmol/L, did not affect cell growth (data not shown).

Effect of retinoids on cell growth. The effects of all-trans- and 9-cis-retinol (all-t-ROL), dissolved in acetone (0.25%), on the growth of MCF-7, Hs578T and MDA-MB-231 human breast cancer cells grown at a density of 5000 cells/cm². Cells were treated with retinol at concentrations of 10 nmol/L, 100 nmol/L and 1 μmol/L in the media for 9 d. Values are means ± SD of three replicate assays; *significantly different from control, P < 0.05.
(P < 0.05) of all-trans-, 9-cis- and 13-cis-retinoic acid were observed on the growth inhibition of MCF-7 cells and growth stimulation of MDA-MB-231 cells. These data are not represented here because similar results have previously been published elsewhere (Marth et al. 1993, Van heusden et al. 1998).

Effect of cell concentration and growth on carotenoid- and retinoid-induced growth inhibition. Several of the growth experiments were also conducted at high and low initial densities of MCF-7 cells. Figure 4A shows the growth of the cells treated with β-carotene and all-RA, when grown at a high initial density (15,000 cells/cm²). β-Carotene and all-RA at 10 and 1 μmol/L, respectively, did not inhibit growth under these conditions, whereas these concentrations significantly reduced the cell growth when the initial cell density was 5000 cells/cm² (Figs. 1, 4).

In the control cultures of MCF-7 cells, a rapid increase in cell proliferation was observed between d 5 and 9 of growth. The cells grew from ~8 x 10^5 cells/well on d 5 to ~5 x 10^6 cells/well on d 9 (not shown). To determine whether the growth inhibitory effect of all-RA was due to the rapid cell doubling between these two time points, one set of cells was treated with 1 μmol/L all-RA from d 5 onward and compared with another set of cells supplemented with 1 μmol/L all-RA from d 1 onward. The cell numbers in the group supplemented from d 5 onward were not different from the control group (Fig. 4B), indicating that an accumulation of retinoic acid in the cells before this time point is necessary for the retinoid to be effective. The other possibility could be that the cell density on d 5 was too large for the retinoid to have an effect.

Effect of all-RA on the expression of RAR. The expression of RARα and γ in MCF-7 cells as a result of all-RA treatment is shown in Figure 5. Cells were treated with 15 μmol/L all-RA for 2, 6, 8, 24 or 72 h and investigated for the induction of retinoid receptors at the mRNA level using Northern blot analysis. The bands were compared using densitometric scanning after normalizing with the pHE7 control (Table 1). The observed induction of receptors due to retinoid treatment was not found to be consistent in other replicate analyses.

Effect of all-RA on the cell cycle stage distribution. Figure 6 shows the cell cycle distribution of Hs578T cells treated with 1 μmol/L all-RA for 6 and 9 d. A significant increase in cell number in the G0/G1 stage of the cell cycle after treatment with all-RA was observed for d 6 and 9. No increase was observed for MCF-7 cells under similar conditions (data not shown).

![FIGURE 4](image-url) Effect of an alteration in culture and treatment conditions on carotenoid- and retinoid-induced growth inhibition of MCF-7 human breast cancer cells. (A) Cells were grown at a density of 15,000 cells/cm² and treated with either 10 μmol/L β-carotene (BC) or 1 μmol/L all-trans-retinoic acid (All-RA) in the media for 9 d. (B) Cells were grown at a density of 5000 cells/cm² and treated with 1 μmol/L retinoic acid supplied from d 1 onward (hatched bar) or d 5 onward (open bar) in the media for 9 d. Values are means ± SD of three replicate assays; *significantly different from control, P < 0.05. Abbreviation: THF, tetrahydrofuran.

![FIGURE 5](image-url) Northern blot analysis of the expression of retinoic acid receptors α and γ (RARα and γ) in MCF-7 and RARα, β and γ in Hs578T human breast cancer cells treated with 1 μmol/L all-trans-retinoic acid (RA) dissolved in acetone (0.25%) for 2, 6, 8, 24 and 72 h. The fold induction of the receptors is shown in densitometric scanning of the bands after normalizing to pHE7 (Table 1). The observed induction of receptors due to retinoid treatment was not found to be consistent in other replicate analyses.
TABLE 1
Fold induction of retinoid receptor subtypes in MCF-7 and Hs578T cells treated with all-trans-retinoic acid

<table>
<thead>
<tr>
<th>Band2</th>
<th>2 Hour</th>
<th>6 Hour</th>
<th>8 Hour</th>
<th>24 Hour</th>
<th>72 Hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAR α</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper band</td>
<td>1.24</td>
<td>0.87</td>
<td>1.13</td>
<td>1.3</td>
<td>1.43</td>
</tr>
<tr>
<td>Lower band</td>
<td>1.13</td>
<td>0.86</td>
<td>1.15</td>
<td>1.44</td>
<td>1.7</td>
</tr>
<tr>
<td>RAR γ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper band</td>
<td>1.23</td>
<td>0.88</td>
<td>1.1</td>
<td>1.47</td>
<td>2.01</td>
</tr>
<tr>
<td>Hs578T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAR α</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper band</td>
<td>1.48</td>
<td>0.97</td>
<td>1.08</td>
<td>1.56</td>
<td>0.58</td>
</tr>
<tr>
<td>Lower band</td>
<td>1.77</td>
<td>0.72</td>
<td>0.97</td>
<td>1.52</td>
<td>0.57</td>
</tr>
<tr>
<td>RAR β</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper band</td>
<td>1.44</td>
<td>1.24</td>
<td>1.34</td>
<td>1.53</td>
<td>0.67</td>
</tr>
<tr>
<td>Lower band</td>
<td>1.26</td>
<td>1.34</td>
<td>1.53</td>
<td>1.92</td>
<td>1.1</td>
</tr>
<tr>
<td>RAR γ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper band</td>
<td>1.44</td>
<td>1.01</td>
<td>1.12</td>
<td>1.86</td>
<td>0.41</td>
</tr>
<tr>
<td>Lower band</td>
<td>1.42</td>
<td>1.42</td>
<td>1.12</td>
<td>1.48</td>
<td>0.34</td>
</tr>
</tbody>
</table>

1 MCF-7 and Hs578T cells were treated with 1 μmol/L all-trans-retinoic acid for 2, 6, 8, 24 or 72 h and investigated for the induction of retinoid receptors (RARα, β and γ) at the mRNA level using Northern blot analysis (Fig. 5).

2 The density of the bands was determined using densitometric scanning. The values obtained were normalized with the values obtained for pHE7 control to determine the fold induction under retinoid treatment.

3 A small induction of the retinoid receptor subtypes was observed at some time points, but this induction was not consistent.

Effect of all-t-RA on the induction of apoptosis. Chromatin condensation was used as the criterion to determine the induction of apoptosis in the cells as a result of all-t-RA treatment. MCF-7 cells did not contain fragmented nuclei, a marker of apoptosis, when treated with 1 and 10 μmol/L all-trans-retinoic acid at 1 and 10 μmol/L concentration for 7 d.

TABLE 2
Cell death induced by all-trans-retinoic acid (all-t-RA) in MCF-7 cells

<table>
<thead>
<tr>
<th>Green cells2</th>
<th>Orange cells2</th>
<th>Orange cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 200 cells</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Control3</td>
<td>195.00 ± 2.65</td>
<td>5.00 ± 2.65</td>
</tr>
<tr>
<td>All-t-RA3,1</td>
<td>161.67 ± 10.02</td>
<td>38.33 ± 10.02</td>
</tr>
<tr>
<td>All-t-RA3,2</td>
<td>149.33 ± 15.31</td>
<td>50.67 ± 15.31</td>
</tr>
</tbody>
</table>

1 MCF-7 cells were treated with all-trans-retinoic acid at 1 and 10 μmol/L concentration for 7 d.

2 Cells were stained with acridine orange and propidium iodide (25 μg/mL) and studied under fluorescence microscope. Orange cells with no nuclear fragmentation were considered dead and counted against live green cells as a marker for necrosis.

3 Values are means ± SD of three replications with 200 cells counted in each replication; * retinoid-induced cell death was significant (P < 0.05) and dose dependent.

DISCUSSION

Numerous studies have been conducted in recent years to evaluate the potential role of vitamin A in the prevention of breast cancer (Hunter and Willett 1994). The results of both case-control and prospective studies have shown a risk reduction associated with diets high in vitamin A. Retinoids including all-trans-, 9-cis- and 13-cis retinoic acid have been studied by various investigators using cancer cells in culture as model systems of breast cancer (Prakash et al. 2000). Few investigators have tried to study the role of other retinoids, and to an even lesser extent, carotenoids, for their effects on breast cancer cell growth in culture.

The growth assays conducted in this study were based on the time required for control cultures to reach confluency (d 9) at an initial seeding density of 5000 cells/cm². Until d 9, cells demonstrated normal cell morphology as determined by phase contrast microscopy. Beyond this point, a change in the cell morphology and a rapid increase in cell detachment were observed. Therefore, d 9 was determined to be the point of termination of these growth studies. The cells were counted in both control and treatment cultures on d 2, 5 and 9. The cells that die during culture detach from the plastic of the culture plates and are automatically removed when the growth medium is changed. Hence, the cells counted at these time points were actually the proliferating viable cells attached to the plates. The results demonstrate that the number of these proliferating viable cells was altered in treatment groups compared with control groups on d 9 of treatment. The inhibitory treatments led to a more cell detachment from the plastic and hence a lower cell count. Growth inhibition was observed to be maximal at d 9 of treatment; therefore the data are shown for this time point only.

Tetrahydrofuran (0.1%) and acetone (0.25%) were used in

FIGURE 6 Effect of all-trans-retinoic acid (All-t-RA) on the cell cycle distribution of Hs578T cells. Cells were treated with the retinoid at a 1 μmol/L concentration for up to 9 d. Values are means ± SD of three replicate assays; * significantly different from control, P < 0.05.
this study to deliver carotenoids and retinoids to the cells and were not found to be toxic to the cells (data not shown). Carotenoids and retinoids were supplied to the cells every other day in this study. Other investigations, however, have used from 1 (Zhu et al. 1997) to 4 d (Shultz et al. 1992) as the time period between feeding the cells. Similarly, the entire duration of treatment before determining the effects on cell growth also differs among studies. The reported duration of treatment has varied from 3 (Kaleagasioglu et al. 1993) to 13 d (Shultz et al. 1992).

β-Carotene at 10 μmol/L is similar to the concentration found in the sera of humans supplemented with β-carotene (Nierenberg et al. 1991). In general, concentrations of 1 and 10 μmol/L for retinoids and carotenoids, respectively, resulted in maximum inhibition of cell growth in this study. This is consistent with other studies in which retinoids have been used at varying levels, with the 1 μmol/L dose resulting in maximum growth cessation (Chen et al. 1997, Fanjul et al. 1996, Marth et al. 1993, Van heusden et al. 1998, Zhao et al. 1995, Zhu et al. 1997). The growth inhibitory effect of lycopene in MCF-7 cells (at 10 μmol/L) observed in this study, however, was markedly less than that observed by Levy et al. (1995) (0.3 μmol/L), possibly due to the different culture conditions and the assay used.

A significant effect (P < 0.05) of all-trans- and 9-cis-retinoic acid was observed on the growth inhibition of Hs578T cells (Fig. 2). Similarly, there was a significant effect (P < 0.05) of all-trans-, 9-cis- and 13-cis-retinoic acid on the growth inhibition of MCF-7 cells and growth stimulation of MDA-MB-231 cells. These data are not presented here, however, because similar results have previously been published elsewhere (Marth et al. 1993, Van heusden et al. 1998). On the basis of a report by Repa et al. (1993) that indicated that all-trans-retinol is a ligand for the RAR and only four- to sevenfold less potent than all-t-RA in binding to RAR(α, β and γ) proteins in competitive binding assays, we set out to test the efficacy of this compound on the growth of MCF-7, Hs578T and MDA-MB-231 cell lines. We found that a 1 μmol/L dose of all-trans-retinol induced growth inhibition almost similar to that observed with the same dose of all-t-RA in both MCF-7 (not shown) and Hs578T cells.

The initial cell density appears to be of paramount importance in determining the effects of carotenoids and retinoids on cell proliferation. In the series of growth experiments conducted in our laboratory, we found that the cells must be at an appropriate density to be sensitive to retinoid and carotenoid treatments. When the starting cell numbers of MCF-7 cells were increased to ~15,000 cells/cm² (Fig. 4), neither β-carotene nor all-t-RA inhibited growth at concentrations of 10 and 1 μmol/L, respectively. However, significant growth inhibition was achieved using the same levels of these compounds when the starting cell numbers were reduced to almost one third (Figs. 1, 4). We also observed that an uptake of all-t-RA by the cells in the early phase of cell culture is essential for this retinoid to inhibit growth. Cells treated with retinoic acid from the middle phase of culture, i.e., when the cells achieved a count of ~98,000 cells/cm², showed no growth inhibition (Fig. 4).

Among patients with breast cancer, tumors that express estrogen receptors (ER) are associated with improved survival and better response to hormone therapy than those not expressing these receptors (Rutqvist 1990). Despite the general observation that ER(−) breast cancer cells are not sensitive to retinoids (Lacroix and Lippman 1980, van den Burg et al. 1993), the growth of ER(−) Hs578T cells was inhibited by all-trans- and 9-cis-retinoic acid as well as by β-carotene in this investigation. Thus, breast cancer cells may be sensitive to carotenoid and retinoid treatments irrespective of their ER status.

Retinoids exert their modulatory effects on cell growth by binding to the nuclear retinoid receptor proteins, of which there are two classes, the RAR and the retinoid X receptor (RXR), each of which has three subtypes (α, β and γ). Retinoic acid is thought to first bind to its nuclear receptors; this ligand-receptor complex in the form of homodimers or hetero dimers then binds to its respective response elements (retinoic acid response elements) in the promoters of a variety of retinoic acid–responsive genes. Binding of the natural reti noids all-t-RA and 9-cis-retinoic acid to the retinoid receptors enhances receptor responses (Allenby et al. 1993, Heyman et al. 1992, Levin et al. 1992). The RAR bind to both all-t-RA and 9-cis-retinoic acid with high affinity, whereas RXR bind to 9-cis-retinoic acid only (Heyman et al. 1992, Levin et al. 1992). RXR play an extremely important role in mediating retinoic acid and vitamin D effects at the level of gene expression by forming heterodimers with RAR and vitamin D receptor, respectively (Zhao et al. 1995).

The Northern blot analyses revealed that all three cell lines used in this study express basal levels of RARα and γ, whereas only Hs578T cells express RARβ. The level of the basal expression of these receptor subtypes was also variable in the three cell lines. We did not observe a consistent induction of any of the RAR subtypes in MCF-7 and Hs578T cells either at the mRNA level using Northern blot analysis (Fig. 5) or at the protein level using Western blot analysis (data not shown).

Several studies have tried to correlate the retinoid receptors to the retinoid sensitivity, and a majority of these studies have used different receptor-selective retinoids for this purpose. RARα-selective retinoid agonists inhibited the anchorage-dependent growth of MCF-7 cells after a 7-d treatment, suggesting that RARα is the retinoid receptor involved in the inhibition of adherent cell growth by retinoids (Dawson et al. 1995). In another study, retinoic acid strongly inhibited the proliferation of ER(+) MCF-7 cells but not of ER(−) Hs578T and MDA-MB-231 cell lines, and RARα mRNA was highly expressed in the RA-responsive lines, but not in the unresponsive lines (van den Burg et al. 1993). The authors suggested that the loss of functional RAR may be a frequent event, leading to ER unresponsiveness of ER(−) breast cancer cells. RARβ-negative cell lines MCF-7 and MDA-MB-231 underwent growth inhibition associated with G1 arrest when treated with 1 μmol/L all-t-RA after a human RARβ gene was introduced into these cell lines using a retroviral vector-mediated gene transduction (Seewaldt et al. 1995). Similarly, RARβ induction in MCF-7 cells by all-t-RA was demonstrated using different receptor-selective retinoids. By using an RARα-selective antagonist (Ro 41–5253), RARβ expression was induced by all-t-RA through an RARα-dependent signaling pathway in MCF-7 cells (Shang et al. 1999). Receptor-selective retinoids induced apoptosis in MCF-7 cells, indicating that RARα, RARγ and RXRα can mediate programmed cell death (Toma et al. 1998). These observations, which have followed an indirect approach of relating retinoid receptors to the cell growth inhibitory mechanism, suggest the involvement of all three subtypes of RAR in the growth inhibition of breast cancer cells. The results of our study, which involved a direct method of determining the expression of RAR as a result of treatment with their natural ligand all-t-RA, indicate that altering RAR expression is not involved in the growth inhibition of breast cancer cells caused by this retinoid. Similarly, RARγ selectively binding retinoids (CD2325, CD666 and CD437) inhibited the proliferation of MCF-7 cells similar...
to all-TRA and no correlation was found between expression of RAR and antiproliferative effects of the retinoids (Widschwendter et al. 1997).

The flow cytometry results of this investigation indicate an arrest of Hs578T cells in the G0/G1 stage of the cell cycle as a result of all-TRA treatment (Fig. 6). The same effect was not observed for MCF-7 cells. It is possible that all-TRA induces the expression of G0/G1-specific gene(s) in Hs578T cells, which were not assessed in this study. Apoptotic induction as a result of all-TRA treatment was not observed in MCF-7 cells because the morphological nuclear fragmentation did not occur. The use of more sensitive techniques for the determination of early apoptosis, such as Annexin V or BRUD staining methods, may provide greater insight into retinoid-induced apoptosis in these cells. There was, however, a dose-dependent necrotic death observed in MCF-7 cells induced by all-TRA (Table 2) on the basis of the accumulation of fluorescent dyes in the cells (Broadus et al. 1996) as explained in Materials and Methods.

The overall results of our study demonstrate that carotenoids and retinoids inhibit the growth of both ER(+) and ER(−) breast cancer cell lines, indicating that estrogen receptor status is an important, although not essential factor for the responsiveness of breast cancer cells to carotenoid and retinoid treatments. Also, the mechanism behind growth inhibition of ER(+) and ER(−) Hs578T cells by all-TRA does not involve transcriptional modulation of the RAR by this retinoid. We are convinced, however, that the mode of action of carotenoids and retinoids in breast cancer cells should not be considered to be limited to the enhancement of basal expression of retinoid receptors (both RAR and RXR series) as a result of treatment. Other mechanistic pathways that are either followed by or concomitant with growth inhibition are possible, including the induction of activation protein-1–responsive genes and the cell cycle–specific genes.

LITERATURE CITED


