Retinoic Acid Inhibition of Human Breast Carcinoma Proliferation Is Accompanied by Inhibition of the Synthesis of a M, 39,000 Protein

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ABSTRACT

Retinoic acid (RA) inhibits proliferation of numerous breast carcinoma cells and prevents estrogen stimulation of growth of several estrogen receptor (ER)-positive cell lines. RA inhibition of human breast carcinoma cell proliferation is associated with marked inhibition of the synthesis of a M, 39,000 protein in the ER-positive human breast carcinoma cell lines investigated. Inhibition of the synthesis of the M, 39,000 protein occurred within 24 h of RA addition and coincides with the onset of inhibition of cellular proliferation. Increasing the dose of RA results in increasing inhibition of M, 39,000 synthesis. RA does not inhibit the proliferation of the ER-negative human breast carcinoma cell line MDA MB-231; synthesis and inhibition of the M, 39,000 protein is not noted in this cell line. Tamoxifen, which inhibits ER-positive breast carcinoma proliferation, moderately inhibits M, 39,000 synthesis, while a concentration of difluoromethylornithine which inhibits cellular proliferation by greater than 50% does not affect M, 39,000 protein synthesis. Thus, inhibition of the M, 39,000 protein appears not to be simply related to the cessation of cellular proliferation.

INTRODUCTION

Numerous investigators have demonstrated the ability of retinoids to profoundly affect cellular proliferation, differentiation, and expression of the transformed phenotype (1–3). The growth of numerous transformed cells is markedly inhibited in vitro by the presence of retinoids (4). These have included sarcoma, lymphosarcoma, mammary carcinoma, myeloma, neuroblastoma, and melanoma cell lines. The continuous presence of the retinoid appears to be required for growth inhibition to persist in the majority of the studies. Retinoids have been demonstrated to profoundly inhibit the proliferation of numerous hormone-dependent breast carcinoma cells (5–9). Retinoids have also displayed efficacy in inhibiting the proliferation of carcinogen-induced mammary tumors, the majority of which are hormonally responsive (10–13). The mechanism by which retinoids inhibit the growth of these cells remains unclear.

Breast carcinoma cells have been demonstrated to produce and secrete numerous growth factors, which bind to receptors located on these cells and stimulate their proliferation (14–17). Several investigators have speculated that retinoids may inhibit cellular proliferation by interfering with either growth factor production or growth factor receptor affinity and/or number or interfering with the post-receptor mitogenic cascade (18). RA has been demonstrated to modulate the number and affinity of epidermal growth factor receptors in numerous systems and block the ability of transforming growth factor β to induce a mitogenic response in growth-arrested AKR-2B fibroblasts (19, 20). In this report, we demonstrate retinoid inhibition of the synthesis of a M, 39,000 protein. The inhibition of the synthesis of this protein correlates with retinoid inhibition of cellular proliferation.

MATERIALS AND METHODS

Materials. DMEM, F-12 medium, gentamycin, and fetal bovine serum were obtained from Gibco (Grand Island, NY). RA and TMX were obtained from the Sigma Chemical Co. (St. Louis, MO). DFMO was a gift from Merrel-Dow (Cincinnati, OH). Phenol red-free DMEM was obtained from Hazleton Research Products (Lenexa, KS) and Gibco. Estradiol was obtained from Steraloids, Inc. (Wilton, NH).

[¹⁴C]Methionine (>80 Ci/mmol) was obtained from New England Nuclear (Boston, MA). The MCF-7, ZR-75, T47D, and MDA-MB-231 cells were a gift of Dr. Mark Lippman (National Cancer Institute, Bethesda, MD). BT 474 and MDA-MB-361 were obtained from the American Type Culture Collection (Rockville, MD). RR01, a mutant of MCF-7 resistant to RA, was generated by progressively exposing the cells to increasing concentrations of RA. This mutant possessed a growth rate similar to the wild-type MCF-7 in the absence of RA but was resistant to concentrations of RA as high as 5 μM. Tissue culture 6-well plates (9.75 cm²) were obtained from Corning (Corning, NY).

Cell Culture. The breast carcinoma cell lines were Mycoplasma-free and maintained in DMEM/F-12 (1:1) medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mm glutamine, and 50 μg/ml gentamycin, in a 5% CO₂ humidified atmosphere at 37°C. Viability was determined by trypsin blue exclusion. In growth-inhibition experiments, the cells were seeded in 6-well plates. The cells were incubated for 24 h in DMEM/F-12 medium supplemented as described above. The cells were then washed and incubated in phenol red-free DMEM supplemented with 4% heat-inactivated, charcoal- and sulfatase-treated fetal bovine serum, for 3 h. The cells were then washed and this procedure was repeated an additional 2 times in order to remove estrogenic compounds. The cells were then incubated in a similar medium, in the presence of 10⁻⁸ m estradiol, 1 μM TMX, or a combination of these agents, for 6 days. The media and agents were changed every 48 h. A similar amount of vehicle (0.05% ethanol) was added to the control flasks. This concentration of ethanol has no effect on cell growth or viability.

Labeling and Analysis of Released Proteins. MCF-7, T47D, and ZR-75, BT 474, and MDA-MB-361 cells were seeded at cell concentrations of 1 × 10⁵, 5 × 10⁴, 2 × 10⁴, 1.5 × 10⁴, and 2 × 10³ cells/well, respectively, in DMEM/F-12 (1:1) medium supplemented with 10% heat-inactivated fetal bovine serum and gentamycin (25 μg/ml). The cells were incubated for 24 h, estradiol was removed as previously described, and the cells were incubated in phenol red-free DMEM supplemented with 4% heat-inactivated, charcoal- and sulfatase-treated fetal bovine serum. Cells were labeled by washing the cultures with 1 ml of phenol red-free methionine-free minimal essential medium 4 times and then replacing the medium with 300 μl of this medium containing 100 μCi [¹⁴C]methionine and the appropriate agent, for 6 h. The medium was removed and the adherent cell layer was washed twice with 1 ml of phosphate-buffered saline and then 200 μl of lysis buffer, consisting of 0.5% Nonidet P-40, 10 mM Tris-HCl, pH 7.0, 0.40 kalikrein inactivator units/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. The cells were lysed according to the method of Nilson-Hamilton and Hamilton (21). In brief, the cells were scraped and harvested into a microcentrifuge tube. The contents were vortexed for 10 s and then spun at 16,000 rpm for 10 min in a microfuge. The supernate was collected and the pellet was dissolved in sample buffer, consisting of 2% sodium dodecyl sulfate, 100 mM Tris-HCl, pH 6.8, 20% glycerol, and 2% β-mercaptoethanol. Aliquots of either the super-
Table 1 Cell growth in the presence of RA and estradiol

MCF-7, T47D, ZR-75, and MDA-MB-231 cells were seeded in wells at $1 \times 10^5$, $5 \times 10^5$, $2 \times 10^5$, and $3 \times 10^5$ cells/well, respectively. RA and estradiol were added to final concentrations of 1 µM and 1 nM, respectively. Media and additions were changed every 48 h. Total incubation time was 6 days.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cell no. $\times 10^4$</th>
<th>% of control</th>
<th>Cell no. $\times 10^4$</th>
<th>% of control</th>
<th>Cell no. $\times 10^4$</th>
<th>% of control</th>
<th>Cell no. $\times 10^4$</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.3 ± 0.9*</td>
<td>1.7 ± 0.5</td>
<td>6.6 ± 1.0</td>
<td>2.7 ± 0.5</td>
<td>2.3 ± 0.4</td>
<td>6.0 ± 0.9</td>
<td>3.4 ± 0.6</td>
<td>82 ± 15</td>
</tr>
<tr>
<td>RA</td>
<td>1.7 ± 0.5</td>
<td>51 ± 14</td>
<td>2.3 ± 0.4</td>
<td>82 ± 15</td>
<td>54 ± 14</td>
<td>140 ± 21</td>
<td>80 ± 17</td>
<td>103 ± 12</td>
</tr>
<tr>
<td>Estradiol</td>
<td>6.6 ± 1.0</td>
<td>1.7 ± 0.5</td>
<td>6.0 ± 0.9</td>
<td>2.7 ± 0.5</td>
<td>1.7 ± 0.5</td>
<td>6.6 ± 0.9</td>
<td>1.7 ± 0.5</td>
<td>6.6 ± 0.9</td>
</tr>
<tr>
<td>RA/estradiol</td>
<td>6.6 ± 1.0</td>
<td>2.3 ± 0.4</td>
<td>6.0 ± 0.9</td>
<td>3.4 ± 0.6</td>
<td>5.0 ± 0.8*</td>
<td>7.4 ± 1.2</td>
<td>3.9 ± 0.7</td>
<td>6.4 ± 0.7*</td>
</tr>
</tbody>
</table>

* Mean of seven experiments ± SD.

nate or the pellet, representing 200,000 dpm, were added to either 2x or 1x sample buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis upon 10% acrylamide gels, using the system of Laemmli (22). The gels were dried and exposed to Kodak AR films. The amount of $[^{35}S]$methionine incorporated into the various bands was quantified by scanning the film with a LKB Gelscan XL laser densitometer and determining the area under the various peaks.

RESULTS

RA inhibits the proliferation of the ER-positive MCF-7, ZR-75, and T47D cells but has no effect on the proliferation of the ER-negative MDA-MB-231 cells (Table 1). In addition, RA inhibits estradiol stimulation of proliferation of the ER-positive cells (Table 1). Antiestrogens, which also inhibit the proliferation of ER-positive cells, modulate the synthesis and secretion of numerous growth factors and proteins (23–25). We, therefore, investigated the ability of retinoids to modulate the synthesis of specific proteins in the ER-positive cells and correlated RA inhibition of cellular proliferation with the ability of this agent to modulate the synthesis of specific proteins. RA markedly inhibited the synthesis of a cytosolic M, 39,000 protein in MCF-7 cells (Fig. 1). Inhibition of the synthesis of this protein by RA occurred in both the absence and presence of estradiol (Fig. 1). Examination of the pellet fractions of either untreated, RA-treated, estradiol-treated, or RA- and estradiol-treated cells did not reveal the presence of the M, 39,000 protein (data not shown).

Fig. 1. RA inhibition of M, 39,000 protein synthesis in MCF-7 cells. MCF-7 cells were seeded at a concentration of $1 \times 10^5$ cells/well in DMEM/F-12 medium and washed and grown in phenol red-free medium, as described in “Materials and Methods.” RA and estradiol (EST) were added to a final concentration of 1 µM and 1 nM, respectively. Control wells (CON) contained an equal concentration of ethanol. Media and contents were changed every 2 days. Total exposure time to RA was 6 days. Labeling of the cells with $[^{35}S]$methionine and electrophoresis were performed as described in “Materials and Methods.”

Fig. 2. RA inhibition of M, 39,000 protein synthesis in T47D and ZR-75 cells. T47D and ZR75 cells were seeded in DMEM/F-12 medium and washed and grown in phenol red-free medium, as described in “Materials and Methods.” RA and estradiol (EST) were added to a final concentration of 1 µM and 1 nM, respectively. Control wells (CONT) contained an equal concentration of ethanol. Media and contents were changed every 2 days. Labeling of the cells with $[^{35}S]$methionine and electrophoresis were performed as described in “Materials and Methods.”
Bronzert et al. (24) and Sheen and Katzenellenbogen (25) have demonstrated that antiestrogens stimulate the secretion of M, 39,000 and 37,000 proteins, respectively, in the ER-positive MCF-7 cells. We speculated that RA may perhaps enhance secretion of the M, 39,000 protein and, therefore, its cellular depletion. We examined the media for evidence of secretion of a M, 39,000 protein but found no evidence for secretion of this protein (data not shown).

We investigated whether the synthesis of the M, 39,000 protein is modulated by RA in other human breast carcinoma cell lines. In both ER-positive cell lines ZR-75 and T47D, RA inhibition of the synthesis of the M, 39,000 protein was observed (Fig. 2). Inhibition of M, 39,000 protein synthesis was also observed in two additional ER-positive RA-sensitive cell lines, MDA-MB-361 and BT 474, the growth of both of which is inhibited by approximately 50% in the presence of 1 μM RA (Fig. 3). Synthesis of this protein was not noted either in a mutant of MCF-7 designated RR01, selected for resistance to the antiproliferative effects of RA even at concentrations of 5 μM, or in the ER-negative MDA-MB-231 cells (Figs. 4 and 5).

The ability of varying concentrations of RA to inhibit the synthesis of the M, 39,000 protein was investigated. Increasing concentrations of RA specifically inhibited the synthesis of this protein (Fig. 6). As little as 30 nM RA resulted in a significant decrease in the percentage of radioactivity incorporated in the M, 39,000 band (Table 2). Increasing doses of RA resulted in further inhibition, with 100 nM RA resulting in an 80% reduction in the activity incorporated in the M, 39,000 band. This inhibitory effect of RA was specific for the M, 39,000 protein, as demonstrated by the minimal effect of increasing doses of RA on the radioactivity incorporated into the M, 42,000 band, most likely representing actin (Table 2). Inhibition of the synthesis of the M, 39,000 protein with increasing doses of RA appears to correlate with inhibition of cellular proliferation (Table 2).

Exposure of MCF-7 cells to 1 μM RA results in a significant inhibition of proliferation by 48 h of exposure (Table 3). We investigated whether RA inhibition of the synthesis of the M, 39,000 protein occurred at the time of inhibition of cellular proliferation, perhaps suggesting a role for RA modulation of this protein in RA inhibition of cellular proliferation (Table 3). RA inhibition of M, 39,000 protein synthesis was noted by 48 h of exposure, with greater than 90% inhibition of M, 39,000 protein synthesis (Table 3). This inhibition of protein synthesis correlated with the onset of inhibition of cellular proliferation.
inhibition of cellular proliferation. Similar inhibition of the synthesis of the M, 39,000 protein was noted in the ER-positive breast carcinoma cell lines T47D, BT 474, MDA-MB-231, and ZR-75, the growth of all of which is inhibited by retinoids.

Fig. 5. RA inhibition of M, 39,000 protein synthesis in MDA-MB-231 cells. MDA-MB-231 cells were seeded at a concentration of 3 x 10^4 cells/well in DMEM/F-12 medium and washed and grown in phenol red-free medium, as described in “Materials and Methods.” RA and estradiol were added to concentrations of 1 μM and 1 nm, respectively. The media and contents were changed every 2 days. Cells were incubated for a total of 6 days. Labeling of the cells with [35S]methionine and electrophoresis were performed as described in “Materials and Methods.” CONT, control.

and was specific for the M, 39,000 protein synthesis, as indicated by the minimal effect on M, 42,000 protein synthesis (Table 3). The effects of other inhibitors of cellular proliferation on M, 39,000 protein synthesis were also investigated, utilizing the antiestrogen tamoxifen and the ornithine decarboxylase inhibitor DFMO; both of these agents arrest cellular proliferation but do not cause cell death (26, 27). The addition of 1 μM TMX, which inhibited cellular proliferation by 50%, resulted in a 40% inhibition of M, 39,000 protein synthesis (Fig. 7), while the addition of 5 μM DFMO, which resulted in a similar inhibition of growth (52%), did not affect M, 39,000 protein synthesis (Fig. 8). Thus, inhibition of M, 39,000 protein synthesis does not appear to be simply a result of the cessation of cellular proliferation.

DISCUSSION
These studies demonstrate the ability of RA to inhibit the synthesis of a M, 39,000 cellular protein. Inhibition of the synthesis of this protein correlates with retinoid inhibition of ER-positive breast carcinoma cell proliferation and occurs in both the presence and absence of estradiol. Increasing doses of RA result in increasing inhibition of cellular proliferation. Retinoid inhibition of the synthesis of the M, 39,000 protein occurs within 24 h of retinoid addition and at the time of RA

Table 2. Inhibition of MCF-7 proliferation and synthesis of the M, 39,000 protein by retinoic acid

<table>
<thead>
<tr>
<th>RA concentration (μM)</th>
<th>Cell count × 10^5</th>
<th>% Inhibition</th>
<th>Relative area (%)</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>13.0 ± 2.0</td>
<td>2.74</td>
<td>18.8</td>
<td>2.80</td>
</tr>
<tr>
<td>10</td>
<td>6.5 ± 1.0</td>
<td>14 ± 2</td>
<td>2.36</td>
<td>16.6</td>
</tr>
<tr>
<td>30</td>
<td>9.2 ± 0.8</td>
<td>18 ± 8</td>
<td>2.04</td>
<td>13.6</td>
</tr>
<tr>
<td>100</td>
<td>9.2 ± 0.2</td>
<td>30 ± 2</td>
<td>0.55</td>
<td>4.8</td>
</tr>
<tr>
<td>300</td>
<td>10.7 ± 1.0</td>
<td>30 ± 6</td>
<td>0.53</td>
<td>3.6</td>
</tr>
<tr>
<td>1000</td>
<td>11.2 ± 0.3</td>
<td>50 ± 8</td>
<td>0.26</td>
<td>1.8</td>
</tr>
</tbody>
</table>

\* Area under peak.
\* Percentage of relative area.
Synthesis of the \( M, 39,000 \) protein was not observed in the ER-negative MDA-MB-231 cell line and a mutant of MCF-7, proliferation of both of which is not inhibited by RA. Synthesis of the \( M, 39,000 \) protein was moderately inhibited (40\%) by 1 \( \mu M \) tamoxifen but DFMO, while inhibiting cellular prolifera-

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Cell count ( \times 10^7 )</th>
<th>Inhibition</th>
<th>Area*</th>
<th>Relative area (%)*</th>
<th>Area</th>
<th>Relative area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.4 ± 0.7</td>
<td></td>
<td>6.1</td>
<td>6.7</td>
<td>10.2</td>
<td>11.2</td>
</tr>
<tr>
<td>24</td>
<td>6.9 ± 0.3</td>
<td>7 ± 4</td>
<td>5.6</td>
<td>6.3</td>
<td>10.5</td>
<td>11.8</td>
</tr>
<tr>
<td>48</td>
<td>5.1 ± 0.3</td>
<td>32 ± 4</td>
<td>ND*</td>
<td>ND</td>
<td>9.1</td>
<td>12.3</td>
</tr>
<tr>
<td>72</td>
<td>4.2 ± 0.2</td>
<td>43 ± 3</td>
<td>ND</td>
<td>ND</td>
<td>9.6</td>
<td>11.8</td>
</tr>
<tr>
<td>96</td>
<td>3.6 ± 0.2</td>
<td>51 ± 3</td>
<td>ND</td>
<td>ND</td>
<td>9.3</td>
<td>14.1</td>
</tr>
<tr>
<td>120</td>
<td>2.5 ± 0.7</td>
<td>66 ± 9</td>
<td>ND</td>
<td>ND</td>
<td>9.4</td>
<td>12.8</td>
</tr>
<tr>
<td>144</td>
<td>2.5 ± 0.4</td>
<td>66 ± 5</td>
<td>ND</td>
<td>ND</td>
<td>9.3</td>
<td>10.8</td>
</tr>
<tr>
<td>TMX</td>
<td></td>
<td></td>
<td>3.8</td>
<td>4.4</td>
<td>9.7</td>
<td>10.3</td>
</tr>
</tbody>
</table>

\* Area under peak.
\* Percentage of relative area.
\* ND, not detectable.

The mechanism by which retinoids inhibit cellular proliferation is unclear. High levels of cRABP have been demonstrated in a majority of human breast carcinoma tissues (6, 28, 29). Numerous investigators have proposed that the cRABP is intimately involved as a mediator of retinoid action. This, however, remains controversial. Although several investigators have demonstrated a positive correlation between binding of retinoids to cRABP and their biological actions in various tissues, there appears not to be a correlation between the level of cellular binding proteins and susceptibility to retinoid-induced growth inhibition (30–33). In addition, several cell lines which are sensitive to the antiproliferative effects of these agents do not possess cRABP (34, 35). A complementary DNA has recently
been cloned which encodes a protein that binds RA with high affinity (36, 37). This protein appears to be a ligand-dependent DNA-binding protein which may be a necessary intermediary for retinoid action.

The ability of retinoids to modulate the synthesis of numerous proteins in a variety of systems has been demonstrated (38). Most evidence supports a primary role of retinoids in regulating gene expression (38). The ability of retinoids to suppress the expression of several oncogenes which may play an essential role in cellular proliferation and differentiation has been demonstrated (39, 40). Dean et al. (41) have demonstrated marked post-transcriptional regulation of c-myc levels during F-9 teratocarcinoma stem cell differentiation.

One can speculate that the M, 39,000 protein observed in the breast carcinoma cells is intimately involved in the regulation of cellular proliferation; marked inhibition of its synthesis by retinoids would, therefore, result in inhibition of cellular proliferation. Support for this hypothesis requires the isolation and purification of this protein and delineation of its effect on cellular proliferation.

REFERENCES

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