Human Breast Cancer Cells and Normal Mammary Epithelial Cells: Retinol Metabolism and Growth Inhibition by the Retinol Metabolite 4-Oxoretinol

Anne C. Chen, Xiaojia Guo, Fadila Derguini, and Lorraine J. Gudas

Department of Pharmacology, Cornell University Medical College, New York, New York 10021 [A. C. C., X. G., L. J. G.], and Immunology Program, Memorial Sloan-Kettering Cancer Center, New York, New York 10021 [F. D.]

ABSTRACT

To understand the signaling and growth-inhibitory effects of retinoids, we have examined the metabolism of [3H]retinol in a number of estrogen receptor-positive (ER+) and estrogen receptor-negative (ER−) human breast cancer cell lines. We have also assayed the metabolism of [3H]retinol in normal human mammary epithelial cells. The ER+ breast cancer cell lines MCF-7 and T47D produce [3H]4-oxoretinol from [3H]retinol; the production of [3H]4-oxoretinol is increased by initial culture in the presence of nonradioabeled retinoic acid (RA) or N-(4-hydroxyphenyl)retinamide, indicating that these drugs enhance [3H]retinol metabolism to [3H]4-oxoretinol. No metabolism of [3H]retinol to [3H]RA in these ER+ tumor lines was detected. ER− breast cancer lines MDA-MB-231, MDA-MB-468, and BT20 do not metabolize [3H]retinol to [3H]4-oxoretinol. In the ER− tumor lines, most of the [3H]retinol remains unmetabolized during the 24-h culture period; MDA-MB-468 and BT20 metabolize some [3H]retinol to [3H]RA. Unlike the majority of the tumor lines, the normal human breast epithelial cell strain AD074 and MCF10A rapidly metabolize [3H]retinol to [3H]retinyl esters. No detectable [3H]RA is produced from [3H]retinol in AD074 and MCF10A cells. Thus, the normal breast epithelial strains, the ER+ tumor lines and the ER− tumor lines differ greatly in their pathways of [3H]retinol metabolism. The levels of cellular retinol binding protein-I mRNA expression are not correlated with the levels or types of various retinol metabolites. Whereas the normal breast epithelial cells and the ER+ tumor lines are growth inhibited by RA, N-(4-hydroxyphenyl)retinamide, and 4-oxoretinol, only the 4-oxoretinol is growth inhibitory in the ER− tumor lines. The cellular retinoid acid-binding protein II mRNA levels are not correlated with the growth inhibition by RA or 4-oxoretinol in the normal and tumor lines.

INTRODUCTION

Retinol (vitamin A) and its natural and synthetic derivatives are known as retinoids. These signaling molecules are required for the proper functioning of a large number of cell types with respect to processes such as cell differentiation and the control of cell growth arrest (reviewed in Ref. 1). Retinoids can also act as cancer preventive agents and are presently being used successfully to treat some types of cancer (reviewed in Refs. 2–4). The biological and transcriptional effects of retinoids are generally mediated by their interactions with the RARs and RXRs (reviewed in Refs. 5 and 6). RARs and RXRs have recently provided evidence that RAR ß mediates the growth-inhibitory effects of RA on human breast cancer cells (21, 25–27).

It is intriguing that the growth-inhibitory response to retinoic acid generally appears to correlate with expression of another member of the nuclear hormone receptor family, the ER. ER+ cell lines are generally growth inhibited by retinoids to a greater extent than are ER− breast cancer cell lines (22, 28, 29). ER+ breast cancer cell lines generally express high levels of RAR α mRNA. Estradiol itself can induce the expression of the RAR α isoform, and this leads to increased RA sensitivity with respect to growth inhibition (30, 31). This estradiol up-regulation of the human RAR α gene in breast carcinoma cells is mediated via a half-palindromic estrogen response element and several SP1 motifs (32). When retinoid-resistant, ER− human breast cancer cells are transfected with an expression vector containing the full-length RAR α cDNA, these cells then acquire sensitivity to growth inhibition by retinoids (29). Ectopic expression of the RAR α cDNA also leads to a higher level of RAR β expression (26). In addition, sensitivity to the growth-inhibitory actions of RA can be restored in the estrogen-independent mammary carcinoma cell lines by the introduction of an RAR β expression vector (25–27). The mechanism by which retinoids inhibit the growth of estrogen-dependent mammary carcinoma cells and why this inhibitory effect is reduced or lost in estrogen-independent mammary carcinoma cells are not well understood. To date, it can be stated that RARs are clearly involved in this mechanism (33).

The ligands for the RARs and RXRs have generally been thought to be acid derivatives of retinol. RA binds to the RARs with high affinity, and 9-cis-RA binds and activates both the RXRs and the RARs (reviewed in Refs. 5 and 6). Retinol has been shown to be metabolized to RA in different types of cultured cells and in various tissues (34, 35). However, the fact that retinol is metabolized into such a large number of different compounds (36–38), some of which have only recently been identified (39–42), implies that other metabolites of retinol may be biologically active with respect to receptor activation. We have recently identified a novel endogenous retinol metabolic pathway that is activated during the differentiation of murine F9 teratocarcinoma stem cells (43). This metabolic pathway leads to the production of large quantities of all-trans 4-oxoretinol, which we have shown to be a biologically active retinol metabolite; 4-oxoretinol activates the RARs but does not transcriptionally activate the RXRs (43). A related compound, 4-oxoretinaldehyde, was identified as the major retinoid present in Xenopus embryos (44). In addition to the endogenously produced metabolites of retinol, 4-HPR, a synthetic retinoid, is presently being used in several clinical trials (45–49). The mechanism of
action of 4-HPR is unclear, but relatively high concentrations of this synthetic retinoid are needed to inhibit the growth of mammary carcinoma cells (46, 50–53).

In the series of studies described here, we have examined the metabolism of [3H]retinol in a number of ER+ and ER− human breast cancer cell lines. We have also examined the metabolism of [3H]retinol in normal human mammary epithelial cells. We have determined the effects of initial culture in either RA or 4-HPR on retinol metabolism. From our studies, we have observed that the ER+ breast cancer cell lines generally produce 4-oxoretinol from retinol, and that the normal cultured breast epithelial cells metabolize [3H]retinol differently from the majority of the ER+ and ER− breast tumor cell lines. We have also found that, unlike RA, 4-oxoretinol can inhibit the growth of both ER+ and ER− breast tumor lines. These experiments provide new information concerning the metabolism of retinoids by normal versus tumorigenic cells that should be useful in the design of retinoids for cancer prevention and treatment.

MATERIALS AND METHODS

Cell Culture and Reagents. The normal human mammary epithelial cell line, MCF-10A, and all other human breast cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD). AD074 normal human mammary epithelial cells (specimen identification number 184) were kindly provided by Dr. Martha Stammer (Lawrence Berkeley Laboratories, Berkeley, CA; Refs. 54 and 55). MCF-7, T47D, ZR-75-1, MDA-MB-231, MDA-MB-468, HS578t, and BT20 cells were maintained in DMEM supplemented with 10% heat-inactivated FCS, 2 mM glutamine, and 5 μg/ml insulin at 37°C in 5% CO2. MCF-10A cells were maintained in a 1:1 mixture of Ham’s F-12 medium and DMEM supplemented with 5% horse serum, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, and 20 ng/ml epidermal growth factor (referred to hereafter as 5% HS-F12:DMEM medium). Insulin, hydrocortisone, and epidermal growth factor were obtained from Sigma Chemical Co. (St. Louis, MO). All other cell culture reagents were obtained from Life Technologies, Inc. (Grand Island, NY). AD074 cells were maintained in serum-free MEGM according to the manufacturer’s instructions (Clonetics, San Diego, CA). Cell lines were checked for their estrogen receptor mRNA status by Northern analysis. MCF-7 and T47D were positive for ER mRNA expression; MDA-MB-231, MDA-MB-468, and HS578t were negative (data not shown). RA and estradiol (Sigma Chemical Co.) were dissolved in 100% ethanol and stored at −20°C. [3H]RA was obtained from NEN Dupont (Boston, MA). 4-HPR was obtained from R. W. Johnson Pharmaceutical Institute (Raritan, NJ). In cell growth experiments, cells were cultured in 12- or 24-well dishes. Duplicate dishes were counted at each time point. Drugs were added 12–16 h after plating. 4-oxoretinol was prepared as described by Ackbar et al. (43).

Extraction of Retinoids and HPLC Chromatography. For the experiments involving only human breast cancer cell lines, cells were plated in 60-mm tissue culture dishes at a density of 1 × 10^6 cells/dish in DMEM medium containing 10% heat-inactivated FCS, 2 mM glutamine, and 5 μg/ml insulin. Twenty-four h after plating, 1 × 10^-6 M RA or 1 × 10^-8 M estradiol were added to cells for 48 h. Then, the medium described above, but with 50 nM [3H]ROL (50 Ci/mm), was added to cells for various lengths of time as indicated. For experiments involving AD074 cells and MCF-10A cells, the AD074 cells were plated and cultured in MEGM medium until they reached a density of 5 × 10^6 cells/60-mm dish. At that time, the MEGM medium on AD074 cells was replaced with 5% HS-F12:DMEM, and the cells were maintained in this medium for 48 h prior to the start of the experiment. MCF-10A cells were plated and cultured in 5% HS-F12:DMEM until they reached a density of 5 × 10^6 cells/60-mm dish. AD074 cells and MCF-10A cells were then cultured for an additional 48 h in the absence or presence of 1 × 10^-6 M RA or 1 × 10^-8 M estradiol, followed by culture in the presence of 50 nM [3H]RA or 50 nM [3H]ROL. A separate control consisting of radiolabeled ROL in medium without cells was included in each experiment during the incubation period. Cells and one-quarter of the labeled media were collected. Cells were scraped into ice-cold PBS, and retinoids were extracted immediately with acetonitrile:butanol (50:50 v/v) and saturated dipotassium phosphate as described previously (56, 57). The organic phase containing the labeled retinoids was separated from the aqueous phase by centrifugation. The upper organic phase was collected and dried in a Speed-Vac (Savant). Samples were stored under argon for no longer than 1 week at −70°C.

HPLC analysis was performed using a Waters Millenium system (Waters Corporation, Milford, MA) to separate the various retinoids. Samples were dissolved in ethanol:acetonitrile (1:9 v/v) and applied to an analytical 5-μm reversed-phase C18 column (Vydac, Hesperia, CA) at a flow rate of 1.5 ml/min. Retinoids from samples labeled with [3H]ROL were separated using a gradient from 0% acetonitrile (15 mM ammonium acetate, pH 6.5) to 68% acetonitrile, followed by a shift to 100% acetonitrile. Nonradiolabeled retinoid standards were run concurrently and were monitored at a wavelength of 340 nm on a photodiode array detector (Waters Corporation, Milford, MA), whereas a Packard A-500 radiochromatography detector (Packard Instruments, Downers Grove, IL) was used to monitor the radio-labeled retinoids. For samples in which labeled acid peaks were shifted, 5 μl of diazomethane (Aldrich Chemical Co., Milwaukee, WI) were added to the samples (for a final volume of 100 μl) and incubated for 5 min prior to loading onto the C18 column.

RNA Isolation and Northern Blot Analysis. Total cellular RNA was isolated from cells cultured in the presence or absence of 1 × 10^-6 M RA for 48 h as described by Chen and Gudas (58). RNA was electrophoretically fractionated by size on 1% agarose/2.2 M formaldehyde gels, transferred to nylon filters by blotting, and attached to the filters using a UV Stratalinker 1800. The cDNA probes used in the analysis were labeled with [32P]dCTP using a random primer labeling kit (Boehringer Mannheim) according to the manufacturer’s directions. A mouse CRABP-II probe (58) and an 800-bp PstI fragment containing the entire human CRBP-1 cDNA was excited from pSG-CRBP and was used as a probe (59). A GAPDH cDNA was used to normalize for RNA loading (58).

Biosamples were dehydrated and hybridized at 42°C in 50% (w/v) formamide, 5× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 50 mM NaH2PO4 (pH 7.4), 5 mM EDTA, 0.08% polyvinylpyrrolidone, 10% (w/v) BSA, and 10% (w/v) salmon sperm DNA. After 10–16 h of hybridization, the filters were washed once in 2× SSC, 0.5% SDS for 20 min at room temperature; once in 1× SSC, 0.1% SDS at 60°C; and once in 0.2× SSC, 0.1% SDS at 60°C. Autoradiographs were quantitated using a Phosphorlmager (Molecular Dynamics).

RESULTS

Retinol Is Metabolized to 4-oxoretinol but not to RA in MCF-7 Cells. We examined an ER+ human breast cancer cell line, MCF-7, for its ability to metabolize [3H]retinol. MCF-7 cells were cultured for 48 h in the absence or presence of 1 × 10^-6 M RA and then cultured in the presence of 50 nM [3H]ROL for 24 h. After labeling, cells and media were harvested, retinoids were extracted, and [3H]ROL metabolites were separated by reverse-phase HPLC. Nonradiolabeled retinoid standards were added to each sample to permit the identification of some of the radiolabeled retinoids. The HPLC tracings of both cell and media samples from MCF-7 cells initially cultured in the absence (control) or presence (+RA) of 1 × 10^-6 M RA for 48 h and then in the presence of [3H]retinol for 24 h are shown in Fig. 1. The control and +RA tracings are shown both at full scale and at an expanded scale to permit the visualization of some of the polar [3H]ROL metabolites. After 24 h of culture in the presence of [3H]ROL, MCF-7 cells grown either in the absence or presence of RA had taken up a large amount of [3H]ROL, as indicated by the presence of a large peak in the region of minute 36.8 in the cells that corresponds to ROL (Fig. 1). MCF-7 cells cultured in the absence of RA metabolized approximately 20% of the [3H]ROL during the 24-h period of culture in the presence of [3H]retinol (Fig. 1, control; Table 1). When MCF-7 cells were cultured initially for 48 h in the presence of 1 × 10^-6 M RA and...
then in the presence of [3H]ROL for 24 h, approximately 40% of the [3H]ROL was metabolized, and [3H]ROL was metabolized into a variety of more polar derivatives that were found in both the cells and the medium (Fig. 1, +RA; Table 1). No detectable metabolism of [3H]ROL to [3H]RA was observed in the MCF-7 line (Fig. 1).

Very few retinyl esters were synthesized from [3H]retinol (see the region from min 47–60 in Fig. 1, Cells). The largest peaks that resolved in the region of minutes 15–19 coeluted with unlabeled standards for 4-OHROL and 4-oxoROL. The identification of these peaks was performed by photodiode array analysis (Ref. 43; data not shown). Approximately one-third of the [3H]ROL was metabolized to [3H]4-oxoROL, and some of this was secreted into the medium. MCF-7 cells cultured for 48 h in the presence of $1 \times 10^{-6}$ M RA behaved similarly to control cells (data not shown).

Table 1 Metabolism of [3H]ROL and concentration of [3H]ROL within cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>[3H]ROL remaining after 24 h (nM)</th>
<th>[3H]4-oxoROL synthesized (nM)</th>
<th>[3H]retinyl esters synthesized (nM)</th>
<th>[3H]ROL concentration within cells (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>Control</td>
<td>40</td>
<td>2</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>RA</td>
<td>31</td>
<td>15</td>
<td>&lt;1</td>
<td>7</td>
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<tr>
<td>T47D</td>
<td>Control</td>
<td>42</td>
<td>2</td>
<td>5</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>RA</td>
<td>23</td>
<td>24</td>
<td>&lt;1</td>
<td>9</td>
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<tr>
<td>ZR-75.1</td>
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<td>13</td>
<td>ND$^d$</td>
<td>36</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>RA</td>
<td>6</td>
<td>ND$^d$</td>
<td>42</td>
<td>2</td>
</tr>
<tr>
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<td>Control</td>
<td>50</td>
<td>ND$^d$</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>RA</td>
<td>38</td>
<td>ND$^d$</td>
<td>&lt;1</td>
<td>6</td>
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<td>HS578</td>
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<td>&lt;1</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>RA</td>
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<td>ND$^d$</td>
<td>&lt;1</td>
<td>6</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>Control</td>
<td>33</td>
<td>ND$^d$</td>
<td>9</td>
<td>2</td>
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<td>RA</td>
<td>27</td>
<td>ND$^d$</td>
<td>12</td>
<td>4</td>
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</tbody>
</table>

$^a$ Total [3H]ROL remaining is the sum of [3H]ROL in the cells plus the [3H]ROL in the medium. The starting concentration of [3H]ROL at time 0 is 50 nM.

$^b$ Data are from two experiments with differences that were <15%.

$^c$ RA indicates culture first in the presence of $1 \times 10^{-6}$ M RA for 48 h, followed by culture for 24 h with [3H]ROL.

$^d$ ND, not detected.

ROL Metabolism in ER+ Human Breast Cancer Cell Lines.

We next analyzed a series of ER+ human breast cancer cell lines for their ability to metabolize [3H]ROL. Three ER+ human breast cancer cell lines, MCF-7, T47D, and ZR-75-1, were examined. The cell lines were first cultured in the absence or presence of $1 \times 10^{-6}$ M RA for 48 h and then cultured in the presence of 50 nM [3H]ROL for either 8 or 24 h. Cell and media samples were harvested and analyzed by HPLC. The HPLC tracings for the 24-h cell samples are shown (Fig. 2). The scale for each tracing has been expanded to permit visualization of the [3H] polar metabolites. Two ER+ cell lines, MCF-7 and T47D, behaved similarly with respect to [3H]ROL metabolism (Fig. 2). Cells which were first cultured in the absence of RA (control) did not metabolize much [3H]ROL to polar derivatives, whereas cells that were cultured initially in $1 \times 10^{-6}$ M RA metabolized [3H]ROL to polar derivatives such as [3H]4-oxoROL and [3H]4-OHROL (Fig. 2A; Table 1). Small amounts of various [3H]retinyl esters that elute in the region from minutes 47 to 60 were also produced in T47D cells that were either cultured in the absence or the presence of $1 \times 10^{-6}$ M RA (Fig. 2A). ZR-75-1 cells behaved differently from the other two ER+ cell lines in that [3H]ROL was not metabolized.
RETNOL METABOLISM IN HUMAN BREAST CANCER CELLS

Fig. 2. [3H]ROL metabolism in ER+ and ER− human breast cancer cell lines. Breast cancer cell lines were first cultured in medium containing 10% FCS and 5 μg/ml insulin with or without 1 × 10−6 M RA for 48 h, followed by culture in the presence of 50 nM [3H]ROL for 24 h. Labeled retinoids from cell and media samples were analyzed by reverse-phase HPLC. HPLC tracings of cell samples from control and RA-cultured breast cancer cell lines are presented, with the data plotted as tritiated cpm versus elution time. All data are plotted using an expanded scale for tritiated cpm. Media samples were also analyzed but are not shown. A. HPLC profiles from three ER+ breast cancer cell lines, MCF-7, T47D, and ZR-75-1. B. HPLC tracings from three ER− breast cancer cell lines: MDA-MB-231, HS578T, and MDA-MB-468. An aliquot of the cell sample from MDA-MB-468 cells grown in the presence of 1 × 10−6 M RA (+RA sample) was also incubated for 5 min with diazomethane and then resolved by HPLC. This sample is depicted as +RA (shifted). Unlabeled retinoid standards are shown both before and after treatment with diazomethane. The cold standards are: peak 1, BHT; peak 2, 4-oxoROL; peak 3, all-trans-RA; peak 4, 13-cis-ROL; peak 5, all-trans-ROL; and peak 3′, methyl retinate. This experiment was performed four times with similar results. One experiment is shown.

to polar derivatives in control cells or in RA-treated cells (Fig. 2A). In fact, most of the [3H]ROL that is taken up by the ZR-75-1 cells is metabolized to [3H]retinyl esters. Approximately 20% of the [3H]retinol was metabolized by the MCF-7 and T47D cell lines over the 24-h culture period, whereas ZR-75-1 cells metabolized about 75% of the [3H]retinol (Table 1). Initial culture of the cells in the presence of 1 × 10−6 M RA for 48 h increased the metabolism of [3H]retinol in all three ER+ lines, resulting in a decrease in the total [3H]retinol remaining after 24 h (Table 1). In all samples, the intracellular concentration of [3H]ROL in all three cell lines was much higher than the external concentration (Table 1).

We next wanted to ascertain how [3H]retinol was metabolized in cells over a longer time period than 24 h. For these experiments, cells were cultured in the presence of [3H]ROL (100 nM) for 24 h; then the medium was changed, and culture of the cells was continued in the presence of DMEM and 10% FCS but without [3H]ROL for approximately 7 days. Samples were removed each day for HPLC analysis. When the MCF-7 and T47D cells were examined, we found that small amounts of [3H]4-oxoROL were produced from [3H]ROL over the 7-day time period (data not shown), amounts similar to those seen in the "control" MCF-7 panels in Fig. 1. No additional [3H]retinyl esters were synthesized from the [3H]ROL in the MCF-7 or T47D lines.

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Fig. 3. [3H]ROL metabolism in cultured, normal human mammary epithelial cells. AD074 cells were cultured in defined MEGM medium until 48 h before the start of the experiment, after which time the medium was changed, and cells were cultured in 5% HS-F12:DMEM medium. During the course of the experiment, all human mammary epithelial cell strains and breast cancer cell line cultures were cultured in 5% HS-F12:DMEM medium with or without 1 × 10^-6 M RA for 48 h, followed by culture in the presence of 50 nM [3H]ROL for 24 h. The cell lines examined were two human mammary epithelial cell strains (MCF10A and AD074), and for comparison in the same experiment, an ER- breast cancer cell line (T47D) and an ER- breast cancer cell line (BT20). Labeled retinoids from cell and media samples were analyzed by reverse-phase HPLC. HPLC tracings of cell samples from cells first cultured in the absence (Control) or presence (+RA) of 1 × 10^-6 M RA are presented with the data plotted as tritiated cpm versus elution time. All data are plotted using an expanded scale of tritiated cpm. HPLC tracings for cell samples from cells labeled with [3H]ROL for 24 h are depicted. A separate sample of nonradiolabeled retinoids was added to each sample extracted from MDA-MB-468 cells as well as to an aliquot containing nonradiolabeled retinoid standards. Diazomethane converts RA to methyl retinoate, a more lipophilic compound, which exhibits a different retention time than RA (3'). When the sample from MDA-MB-468 cells was treated with diazomethane, the putative RA peak that eluted at minute 21 was shifted to minute 42 (Fig. 2B, compare MDA-MB-468 + RA sample to + RA (shifted) sample). Similarly, the peak corresponding to RA in the sample containing nonradiolabeled standards was also shifted to minute 42 (Fig. 2B, compare cold standards to cold standards (shifted) sample).

In summary, our results indicate that ER- breast cancer cells metabolize [3H]ROL differently from ER+ breast cancer cells. Unlike even after 5–7 days of culture (data not shown). When the three ER+ cell lines were cultured for 48 h in the presence of estradiol prior to labeling, the HPLC tracings were indistinguishable from those of control cells (data not shown).

Table 2. Metabolism of [3H]ROL and concentration of [3H]ROL within cells

<table>
<thead>
<tr>
<th>Cell line and treatment</th>
<th>Total [3H]ROL remaining after 24 h (nM)</th>
<th>Total [3H]retinyl ester synthesized from [3H]ROL (nM)</th>
<th>Concentration of [3H]ROL within the cells (μM)</th>
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</thead>
<tbody>
<tr>
<td>MCF10A</td>
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<tr>
<td>Control (−RA)</td>
<td>10</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>RA</td>
<td>10</td>
<td>24</td>
<td>2</td>
</tr>
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<td>AD074</td>
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<tr>
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* Data are from two experiments with differences which were <15%.

** RA indicates culture first in the presence of 1 × 10^-6 M RA for 48 h, followed by culture for 24 h with [3H]ROL.
ER+ lines, the ER− breast cancer cells do not generate significant amounts of [3H]4-oxoROL from [3H]ROL. However, in MDA-MB-468 cells, [3H]RA is produced from [3H]ROL.

[3H]ROL Metabolism in Normal Human Mammary Epithelial Cells. We also investigated the metabolism of [3H]ROL in normal cultured breast epithelial cells and compared this metabolism with that in the ER+ and ER− breast cancer cell lines. For the following series of experiments, we used two human mammary epithelial cell strains, AD074 (54, 55) and MCF10A; these cell lines were compared to ER+ T47D cells and to ER− BT20 cells. To examine [3H]ROL metabolism, mammary epithelial cells and breast cancer cells were first cultured for 48 h in the presence or absence of 1 × 10⁻⁶ M RA and then cultured in the presence of 50 nM [3H]ROL for 24 h. The HPLC profiles from the cell samples are shown in Fig. 3 with the scale expanded to better visualize the polar [3H]ROL metabolites. The two normal mammary epithelial cell strains do not metabolize [3H]ROL to more polar derivatives (Fig. 3). In contrast, almost all of the [3H]ROL taken up by these cells is converted into [3H]retinyl esters (Fig. 3; Table 2). No differences are observed in the HPLC profiles between cultures cultured in the presence or absence of RA. As discussed earlier, T47D cells grown under control conditions convert some [3H]ROL to [3H]retinyl esters, whereas T47D cells first treated with 1 × 10⁻⁶ M RA convert a larger portion of the [3H]ROL to [3H]retinyl esters; metabolism of [3H]ROL to polar derivatives, including 4-oxoROL and 4-OHROL, is also observed in the T47D cells (Figs. 2A and 3). The ER− breast cancer cell line BT20 exhibited similar HPLC profiles whether or not the cells were first cultured in the absence or presence of 1 × 10⁻⁶ M RA (Fig. 3) or estradiol (data not shown). BT20 cells (Fig. 5A) resemble the ER-MDA-MB-468 cells (Fig. 2A) in that they convert a small amount of [3H]ROL into a retinoid that coelutes with the standard for RA. It is highly likely that this derivative is RA because the addition of diazomethane to the [3H]RA-treated sample causes a shift due to the conversion of the putative RA to its methyl ester, which elutes at minute 42 (Fig. 3, +RA versus +RA (shifted)).

In summary, our results suggest that normal mammary epithelial cells rapidly metabolize most of the 50 nM [3H]ROL to [3H]retinyl esters. The normal cells synthesize 5-10-fold more [3H]retinyl esters from [3H]retinol than the tumor lines, with the exception of the tumor line ZR-75-1 (Tables 1 and 2). Culture of the normal cells in the presence of RA prior to addition of [3H]ROL had no effect on [3H]ROL metabolism. All of the human breast tumor cell lines, both ER+ and ER− lines, exhibited a decrease in the ability to esterify [3H]retinol to [3H]retinyl esters relative to the normal cells (Fig. 3; Table 2).

Effects of 4-HPR on ROL Metabolism. Because we had observed an increase in [3H]ROL metabolism in the ER+ cancer lines following culture of the cells for a period of time in RA, we wanted to ascertain whether culture in 4-HPR influenced the level of [3H]ROL metabolism or the types of [3H]ROL metabolites produced. Thus, various normal cell strains and tumor lines were cultured for 48 h in the presence of 1 μM 4-HPR followed by culture for 24 h in the presence of 50 nM [3H]ROL (Fig. 4). Two major effects of the treatment of cells with 4-HPR were noted: (a) more [3H]ROL was metabolized by all four cell lines after 48 h of culture in the presence of 1 μM 4-HPR (Fig. 4B); and (b) the 4-HPR treatment increased the synthesis of [3H]4-oxoROL from [3H]ROL 2-10-fold in the ER+ cancer lines MCF-7 and T47D and also in the AD074 normal cell strain; the ER− line MDA-MB-231 did not exhibit any increase in 4-oxoROL production from [3H]ROL after culture in the presence of 4-HPR (Fig. 4A). Thus, 4-HPR acts similarly to RA in that it induces 4-oxoROL production from ROL in the ER+ breast cancer lines. However, at the same external concentration, RA induces much more 4-oxoROL production from ROL than does 4-HPR (Figs. 1, 2A, and 4).

Effects of RA, 4-HPR, and 4-oxoROL on the Growth of the Normal Cell Strain AD074 and the Tumor Lines MCF-7 and MDA-MB-231. The data from the [3H]ROL metabolism studies indicated that in the ER+ tumor lines MCF-7 and T47D, both initial RA and 4-HPR treatment resulted in the induction of metabolism of [3H]ROL to [3H]4-oxoROL. Therefore, we tested whether 4-oxoROL exhibited growth-inhibitory activity in these normal cell strains and tumor lines. Cells were plated either without retinoid or in the presence of either 1 × 10⁻⁷ M RA, 4-oxoROL, or 4-HPR. All three retinoids exhibited growth-inhibitory activity in the normal AD074 cell strain, with 1 × 10⁻⁷ M RA and 4-oxoROL giving somewhat better growth inhibition than 1 × 10⁻⁷ M 4-HPR (Fig. 5A). In the ER− MCF-7 cancer line, both RA and 4-oxoROL were superior to 4-HPR with respect to growth inhibition (Fig. 5A). In the MCF-7 cells, 4-HPR, even at 1 × 10⁻⁶ M, inhibited the growth of the cells by only about 50% (Fig. 5A). In the ER− line MDA-MB-231, neither RA nor 4-HPR was growth inhibitory at the concentrations used in the experiment. However, 4-oxoROL at 1 × 10⁻⁶ M inhibited growth of the MDA-MB-231 cells by approximately 65% (Fig. 5). We also tested the ER+ line T47D and the ER− line MDA-MB-468; T47D cells were greatly growth inhibited by both RA and 4-oxoROL, whereas MDA-MB-468 cells were inhibited by 4-oxoROL but not by RA (Fig. 5B). Thus, in the two ER− lines MDA-MB-231 and MDA-MB-468, 4-oxoROL is superior to both RA and 4-HPR with respect to the inhibition of growth of the cells (Fig. 5).
RETINOL METABOLISM IN HUMAN BREAST CANCER CELLS

A

RA

4-HPR

4-oxoretinol

Fig. 5. A comparison of the growth-inhibitory effects of RA, 4-HPR, and 4-oxoROL. A, cells (1.0 × 10⁴/well) were grown in medium containing 1/1000 ethanol (□, control), 1 μM retinoids (○, 1 μM), or 0.1 μM retinoids (■, 0.1 μM). On days 0–8, cells were trypsinized and counted. The results are plotted as cell number (× 10⁴) per well (Y-axis) versus days in culture (X-axis). Each point is the mean of quadruplicate samples. Bars, SD; no bar, SD < 0.01. B, cells were grown as in A in the presence of 1 μM RA (○), 1 μM 4-oxoROL (■), or control (□).

Changes in Gene Expression in Response to RA and 4-HPR. Because previous reports have suggested that the CRBP-I is involved in ROL metabolism (reviewed in Ref. 34), we examined the levels of CRBP-I mRNA in the various normal and tumor lines (Fig. 6). There was no correlation between the levels of CRBP-I mRNA and the metabolism of [³H]ROL into either [³H]retinyl esters or [³H]RA. For example, T47D, MDA-MB-231, and MDA-MB-468 did not synthesize significant amounts of [³H]retinyl esters from [³H]retinol as compared to the normal cells (Tables 1 and 2), but MDA-MB-468 cells exhibited a high level of expression of the CRBP-I mRNA.
whereas MDA-MB-231 and T47D did not (Fig. 6). Likewise, both the MDA-MB-468 and BT20 lines metabolized [3H]ROL to [3H]RA (Figs. 2 and 3), but only MDA-MB-468 displayed abundant CRBP-I mRNA levels. BT20 CRBP-I mRNA levels were undetectable (Fig. 6).

We also found no correlation between expression of CRABP-II mRNA and sensitivity of cells to growth inhibition by RA, in contrast to a recent report (60). For instance, MCF-7 cells were sensitive to growth inhibition by RA (Fig. 5A), but these cells expressed low levels of CRABP-II mRNA (Fig. 6). MDA-MB-468 cells were resistant to growth inhibition by RA (Fig. 5B) but expressed higher levels of CRABP-II mRNA (Fig. 6). There was also no relationship between CRABP-II mRNA levels and the metabolism of [3H]ROL to [3H]RA. MDA-MB-468 and T47D cells expressed CRABP-II mRNA, whereas BT20 cells did not (Fig. 6). MDA-MB-468 and BT20 cells metabolized [3H]ROL to [3H]RA, whereas T47D did not metabolize [3H]ROL to [3H]RA (Figs. 2 and 3).

**DISCUSSION**

There are several major findings reported here with respect to the metabolism of ROL in normal breast epithelial cells and in various carcinoma lines:

(a) No detectable RA was produced from ROL in the normal cell strains ADO74 and MCF-10A and in the ER+ cell lines MCF-7, T47D, and ZR-75.1. Additionally, ROL was not metabolized into a detectable amount of RA in the ER− carcinoma lines MDA-MB-231 and Hs578t (Figs. 1–3). However, in two of the ER− carcinoma lines, BT20 and MDA-MB-468, a substantial amount of ROL was metabolized to RA (Figs. 2 and 3). Nevertheless, the MDA-MB-468 cell line was not growth inhibited by exogenous RA (data not shown).

(b) A second major point is that, except for the carcinoma line ZR-75.1, very little retinyl ester formation was observed in the tumor lines (Figs. 1–3) compared to the normal cell strains (Fig. 3). In the normal cells strains, ROL was rapidly and extensively metabolized into retinyl esters (Fig. 3; Table 2). We obtained a similar result when we compared squamous cell carcinoma lines from the oral cavity with normal epithelial cell strains from the oral cavity; all of the normal cell strains examined exhibited extensive metabolism of ROL to retinyl esters, whereas the squamous cell carcinoma lines exhibited no or only a small amount of retinyl ester formation from ROL. Similar results were also observed in mammary carcinomas in rats (61). Tumor-bearing animals injected with [3H]ROL exhibited practically no [3H]ROL metabolism in the mammary tumors but extensive esterification in other normal tissues (61).

(c) We observed that two of the three ER+ carcinoma lines, MCF-7 and T47D, when cultured in the presence of exogenous RA for 48 h and subsequently cultured in the presence of [3H]ROL, exhibited the induction of an enzymatic activity that converted ROL into the polar derivative 4-oxoROL (Figs. 1–3; Table 1). In fact, in the T47D line, about one-half of the [3H]ROL added to the cells was metabolized to 4-oxoROL in the 24-h radiolabeling period (Table 1). No detectable 4-oxoROL production from ROL was observed in the four ER− cell lines, MDA-MB-231, MDA-MB-468, BT20, and Hs578t, after culture in the presence of exogenous RA. No detectable 4-oxoROL was produced from ROL in the normal cell strains ADO74 and MCF-10A, presumably in part because the esterification pathway is extremely active in the normal cell strains, as discussed above (Fig. 3).

We have reported previously that 4-oxoROL is biologically active and can transactivate the RARs (43). The fact that the ER+ lines MCF-7 and T47D are also growth inhibited by both RA and 4-oxoROL (Fig. 5) suggests that the mechanism by which exogenously added RA leads to growth inhibition may involve the induction of the metabolism of ROL to 4-oxoROL. Consistent with this idea is our data that the ER− lines MDA-MB-231 and MDA-MB-468, which do not metabolize ROL to 4-oxoROL in response to RA (Figs. 2 and 3), are growth inhibited by exogenous 4-oxoROL but not by exogenously added RA (Fig. 5). Paradoxically, although RA itself can bind to RARs in vitro with high affinity (reviewed in Refs. 5 and 6), this binding to receptors is apparently not sufficient to cause growth inhibition in ER− tumor lines. Thus, RA may inhibit cell growth in part by inducing an enzyme that generates another biologically active retinoid, 4-oxoROL, in cells. 4-oxoROL, via its binding to RARs, may activate different target genes from those activated by RA itself. Furthermore, our data suggest that other carcinoma cells that are unresponsive to RA may be growth inhibited by 4-oxoROL.

The enzyme involved in the oxidation of ROL to 4-oxoROL has not yet been identified. However, several different cytochrome P-450 enzymes are capable of oxidizing ROL, retinaldehyde, and
RA in microsomes (62–74). Evidence indicates that the enzyme(s) involved in ROL oxidation are different from those that mediate RA oxidation (62). However, it is intriguing that cells that metabolize [3H]RA to more polar derivatives are also more sensitive to RA-induced growth inhibition (75). It is likely that only one enzyme converts retinol to 4-OHROL, and 4-OHROL is then converted to 4-oxoROL, but we do not have proof of this yet. We always observe both compounds when MCF-7 or T47D cells are cultured in the presence of 4-HPR or RA, but more 4-oxoROL than 4-OHROL is generally observed.

Our data for the normal breast epithelial cells (Fig. 3; Table 2) is similar to that of Randolph and Simon (36) in that they observed extensive ROL esterification in normal cultured human epidermal keratinocytes. It has been reported previously that the CRBP-I is involved in retinol metabolism, both in the esterification of retinol and in the oxidation of retinol to RA (reviewed in Ref. 34). We did not observe such a correlation between CRBP-I mRNA levels and ROL metabolism, however. For example, the BT20 cell line produced significant amounts of RA from ROL (Fig. 3), and yet this cell line exhibited no detectable CRBP-I mRNA expression (Fig. 6). Conversely, the MDA-MB-468 and T47D lines both exhibited a small amount of ROL esterification (Table 1), but only the MDA-MB-468 line expressed high levels of the CRBP-I message (Fig. 6). We also found no correlation between the tumorigenic phenotype and the loss of CRBP-I mRNA expression, because the tumor line MDA-MB-468 exhibited high CRBP-I mRNA levels (Fig. 6). This conclusion is different from that of Jing et al. (76). Furthermore, no correlation was observed between the expression of the CRABP-II message and either the tumorigenic phenotype or the loss of CRBP-I mRNA expression, because the tumor line MDA-MB-468 exhibited no detectable CRBP-I mRNA expression (Fig. 6). Con

Thus, our data are not in agreement with those recently published by Jing et al. (76). Moreover, no correlation was observed between the expression of the CRABP-II message and either the tumorigenic phenotype or the loss of CRBP-I mRNA expression, because the rat mammary carcinoma cell line MCF-7 exhibited no detectable CRBP-I mRNA expression (Fig. 6). Our data for the normal breast epithelial cells (Fig. 3; Table 2) is similar to that of Randolph and Simon (36) in that they observed extensive ROL esterification in normal cultured human epidermal keratinocytes. It has been reported previously that the CRBP-I is involved in retinol metabolism, both in the esterification of retinol and in the oxidation of retinol to RA (reviewed in Ref. 34). We did not observe such a correlation between CRBP-I mRNA levels and ROL metabolism, however. For example, the BT20 cell line produced significant amounts of RA from ROL (Fig. 3), and yet this cell line exhibited no detectable CRBP-I mRNA expression (Fig. 6). Conversely, the MDA-MB-468 and T47D lines both exhibited a small amount of ROL esterification (Table 1), but only the MDA-MB-468 line expressed high levels of the CRBP-I message (Fig. 6). We also found no correlation between the tumorigenic phenotype and the loss of CRBP-I mRNA expression, because the tumor line MDA-MB-468 exhibited high CRBP-I mRNA levels (Fig. 6). This conclusion is different from that of Jing et al. (76). Furthermore, no correlation was observed between the expression of the CRABP-II message and either the tumorigenic phenotype or the loss of CRBP-I mRNA expression, because the tumor line MDA-MB-468 exhibited no detectable CRBP-I mRNA expression (Fig. 6). Consequently, our data are not in agreement with those recently published by Jing et al. (76). Our data for the normal breast epithelial cells (Fig. 3; Table 2) is similar to that of Randolph and Simon (36) in that they observed extensive ROL esterification in normal cultured human epidermal keratinocytes. It has been reported previously that the CRBP-I is involved in retinol metabolism, both in the esterification of retinol and in the oxidation of retinol to RA (reviewed in Ref. 34). We did not observe such a correlation between CRBP-I mRNA levels and ROL metabolism, however. For example, the BT20 cell line produced significant amounts of RA from ROL (Fig. 3), and yet this cell line exhibited no detectable CRBP-I mRNA expression (Fig. 6). Conversely, the MDA-MB-468 and T47D lines both exhibited a small amount of ROL esterification (Table 1), but only the MDA-MB-468 line expressed high levels of the CRBP-I message (Fig. 6). We also found no correlation between the tumorigenic phenotype and the loss of CRBP-I mRNA expression, because the tumor line MDA-MB-468 exhibited high CRBP-I mRNA levels (Fig. 6). This conclusion is different from that of Jing et al. (76). Furthermore, no correlation was observed between the expression of the CRABP-II message and either the tumorigenic phenotype or the loss of CRBP-I mRNA expression, because the tumor line MDA-MB-468 exhibited no detectable CRBP-I mRNA expression (Fig. 6). Consequently, our data are not in agreement with those recently published by Jing et al. (76). Our data for the normal breast epithelial cells (Fig. 3; Table 2) is similar to that of Randolph and Simon (36) in that they observed extensive ROL esterification in normal cultured human epidermal keratinocytes. It has been reported previously that the CRBP-I is involved in retinol metabolism, both in the esterification of retinol and in the oxidation of retinol to RA (reviewed in Ref. 34). We did not observe such a correlation between CRBP-I mRNA levels and ROL metabolism, however. For example, the BT20 cell line produced significant amounts of RA from ROL (Fig. 3), and yet this cell line exhibited no detectable CRBP-I mRNA expression (Fig. 6). Conversely, the MDA-MB-468 and T47D lines both exhibited a small amount of ROL esterification (Table 1), but only the MDA-MB-468 line expressed high levels of the CRBP-I message (Fig. 6). We also found no correlation between the tumorigenic phenotype and the loss of CRBP-I mRNA expression, because the tumor line MDA-MB-468 exhibited high CRBP-I mRNA levels (Fig. 6). This conclusion is different from that of Jing et al. (76). Furthermore, no correlation was observed between the expression of the CRABP-II message and either the tumorigenic phenotype or the loss of CRBP-I mRNA expression, because the tumor line MDA-MB-468 exhibited no detectable CRBP-I mRNA expression (Fig. 6). Consequently, our data are not in agreement with those recently published by Jing et al. (76).


Human Breast Cancer Cells and Normal Mammary Epithelial Cells: Retinol Metabolism and Growth Inhibition by the Retinol Metabolite 4-Oxoretinol

Anne C. Chen, Xiaojia Guo, Fadila Derguini, et al.


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