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Retinol Conversion to Retinoic Acid Is Impaired in Breast Cancer Cell Lines Relative to Normal Cells

RAFAEL MIRA-Y-LOPEZ,[1]* WEN LI ZHENG,[2] YUVARANI S. KUPPUMBATTI,[1] BRENT REXER,[2] YONGKUI JING,[1] AND DAVID E. ONG[2]

[1]*Department of Medicine, Mount Sinai School of Medicine, New York, New York*
[2]*Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee*

The bioactivity of retinol (vitamin A) is in part dependent on its metabolism to retinoic acid (RA). We investigated the ability of breast epithelial cells to synthesize RA when challenged with a physiological retinol dose (2 μM). Normal human mammary epithelial cells (HMEC) cultured from reduction mammoplasties were competent in RA synthesis and the ability to synthesize RA was retained by immortal, nontumorigenic breast epithelial cell lines (MTSV1.7, MCF-10F, and 184B5). In contrast, most (five of six) breast cancer cell lines could not synthesize RA or did so at low rates relative to normal cells. A notable exception was the MDA-MB-468 cell line, which was fully competent in RA synthesis. Most (≥68%) of the RA synthesized by breast cells was recovered from the culture medium. Cellular retinol binding protein and cellular RA binding protein II, both expressed in HMEC, had various expression patterns in the cell lines that did not correlate with the observed differences in RA synthesizing ability. Strong RA induction of the RA hydroxylase P450RAI (CYP26) was confined to ERα-positive T47D and MCF-7 breast cancer cells and did not appear to explain the lack of detectable RA levels in these cells since RA remained undetectable when the cells were treated with 5–10 μM liarozole, a P450RAI inhibitor. We hypothesize that retinol bioactivity is impaired in breast cancer cells that cannot synthesize RA. In preliminary support of this hypothesis, we found that retinol (0.5–2 μM) inhibited MCF-10F but not T47D or MCF-7 cell growth. J. Cell. Physiol. 185:302–309, 2000.

The biological activities of retinol (vitamin A) are in part dependent on its conversion to metabolites capable of binding and activating members of the RA receptor (RAR)[3] and the retinoid X receptor (RXR) families (reviewed in Kastner et al., 1995). RARs and RXRs mediate the ligand-dependent regulation of target gene transcription through *cis* interactions with RA response elements (RARE) and *trans* interactions with other transcription factors and cofactors. The best characterized active metabolite of retinol is retinoic acid (RA), which, as the all-*trans* isomer, activates RARs and, as the 9-*cis* isomer, activates both RARs and RXRs. Other active metabolites are generated from retinol, such as 4-oxo-retinol, which specifically activates RARs (Lane et al., 1999 and references therein). Although RA is present in the circulation at low levels, most tissues rely on the uptake and metabolism of retinol to achieve RAR and RXR activation (Kurlandsky et al., 1995). Therefore, the regulation of ligand availability is an important aspect of vitamin A biology.

The leukemogenic role of the dominant negative PML–RARα fusion protein (reviewed in Melnick and Licht, 1999) fostered the concept of aberrant RA signaling in cancer, which has been extended to solid human carcinomas with the demonstration of progressive RARβ2 underexpression in cancers of various sites, including the breast (Xu et al., 1997). Recently, several studies have shown that in addition to defects at the

Abbreviations: HMEC, normal human mammary epithelial cells; RA, retinoic acid (unless otherwise specified, the all-*trans* isomer); RAR, retinoic acid receptor; RXR, retinoid X receptor; PML, promyelocytic leukemia gene; ER, estrogen receptor; P450RAI or CYP26, RA hydroxylase; CRBP, cellular retinol binding protein; CRABP, cellular RA binding protein; RARE, RA response element; tk, thymidine kinase promoter; LUC, luciferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ECL, enhanced chemiluminescence; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

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*Correspondence to: R. Mira-y-Lopez, Department of Medicine, Box 1178, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029-6574. E-mail: mira@msvax.mssm.edu

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level of retinoid receptors, cancer is associated with defective retinol processing. Thus, in agreement with earlier work (De Luca et al., 1984; Bhat and Lacroix, 1989), Gudas and coworkers have demonstrated cancer-specific defects in retinol esterification, including in breast cancer (Chen et al., 1997). In addition, there have been limited reports suggesting that RA steady-state levels are also decreased in cancer. Thus, a five- to eight-fold lower RA content was found in prostatic carcinoma relative to normal and benign prostatic tissue (Pasquali et al., 1996), and an anti-RA antibody stained seven of seven specimens of normal oral mucosa but only 20 of 43 premalignant oral lesions (Xu et al., 1995). More recently, expression of the cellular retinol-binding protein I (CRBP) gene, whose product has been implicated in the regulation of retinol homeostasis (Ghyselinck et al., 1999) and metabolism (Ong et al., 1994), was shown to be downregulated in 24% of human breast carcinomas (Kuppumbatti et al., 2000).

We have compared the ability of normal, immortal but nontumorigenic, and tumorigenic human breast epithelial cells to synthesize RA from physiological retinol (2 μM). Our results suggest that malignant breast transformation may be accompanied by a decreased ability to biosynthesize RA.

MATERIALS AND METHODS

Cells

Unless noted otherwise, cells were obtained from and grown as recommended by the American Type Culture Collection (ATCC). ED and EK human breast cancer cells (passage 10–13) were obtained from Dr. Beatriz Pogo (Mount Sinai School of Medicine, New York) and grown as described (Wang et al., 1995). Immortalized but nontumorigenic 184B5 and MTSV1.7 cells were obtained from and grown as recommended by, respectively, Dr. Martha Stampfer (Lawrence Berkeley Laboratory, Berkeley, CA) (Stampfer and Bartley, 1985) and Dr. Joyce Taylor-Papadimitriou (Imperial Cancer Research Fund, London) (Bartek et al., 1991). MCF-10F cells (Soule et al., 1990) were obtained from the ATCC and grown as adherent monolayers in the recommended but normocalcelmic medium. Primary cultures of normal human mammary epithelial cells (HMEC) were generated from residual reduction mammoplasty specimens based on the method of Stampfer (Stampfer et al., 1980). Briefly, the tissue was freed of excess fat, minced with apposed scalpels, and digested overnight at 37°C with 200 U/ml collagenase (type IA, Sigma, St. Louis, MO) and 100 U/ml hyaluronidase (Calbiochem, San Diego, CA). Digestion was continued until epithelial organoids were obtained. After centrifugation and resuspension of the digest, the organoids were separated from single cells by repeated sedimentation at 1 × g. The organoids were then plated in mammary epithelial growth medium (Clonetics, San Diego, CA) supplemented with 5 μg/ml transferin and 10 μM isoproterenol. Following organoid attachment and medium renewal, epithelial outgrowths were allowed to form for ~2 weeks. After brief trypsinization to remove contaminating fibroblasts, the outgrowths were trypsinized and replated in T25 flasks for regrowth and testing.

All cell cultures were allowed to reach near-confluency under normal growth conditions; they were then switched to RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 5 μg/ml insulin and shipped by overnight mail to Vanderbilt University for retinol treatment and HPLC analysis of RA content. Thus, all cells were analyzed under a single medium to minimize any confounding effect that the different optimal growth media might have had.

HPLC analysis of RA

The procedures used for RA analysis have been described in detail (Bucco et al., 1997 and references therein). Briefly, cultures were pulsed with 2 μM all-*trans* retinol/8 μM bovine serum albumin (BSA) for 12 h at 37°C and extracts of the medium and cells resolved by HPLC. All-*trans* RA was identified based on its coelution with authentic all-*trans* RA and was confirmed as such based on its absorption spectrum, as previously described (Bucco et al., 1997). The absorbance maximum of the collected peak was ~350 nm, indistinguishable from the maximum at 352 nm for authentic all-*trans* RA, and addition of base to the sample shifted the maximum to ~335 nm, just as described for authentic all-*trans* RA. The all-*trans* RA peak was quantitated by relating the area under the curve (AUC) to the AUC generated by all-*trans* RA standards and was normalized to total cell protein, which was quantitated by using the BCA protein analysis kit (Pierce, Rockford, IL). Results are expressed as pmols RA/mg of cell protein. Extracts of replicate cultures treated with BSA alone were also run. Under these conditions, some RA might have been detected since the culture medium contained 10% heat-inactivated serum and thus some retinol. However, no RA was detected, indicating that RA levels resulting from the retinol in 10% heat-inactivated serum were below the limit of detection (~1 pmol RA/mg of cell protein). In contrast, easily detectable levels of RA were obtained in a cell-specific manner following supplementation with 2 μM retinol. By necessity, such RA was synthesized from the added retinol. Most of the retinol added to the medium was recovered as retinol itself and RA, when present, amounted to a small percentage (≤1%); because the percent recovery of retinol was in the range of 56–85% (data from MTSV1.7 and MDA-MB-468 cells, each analyzed twice), not all of the retinol input may have been accounted for (note that the recovery of retinol added to cells and immediately extracted was 80–90%). The fraction unaccounted for may have included retinyl esters, other retinol metabolites, RA metabolites, and isomers of either retinol or RA.

Western blot analysis

Cells were homogenized in 10 mM tris(hydroxymethyl)aminomethane (Tris). HCl, 1 mM EDTA, pH 7.4, and cytosolic fractions obtained by centrifugation at 125,000 × g for 60 min at 4°C in a Beckman ultracentrifuge. Immunoblots containing 50 μg of cell protein/lane were analyzed by enhanced chemiluminescence as described (Zheng et al., 1996) by using previously characterized antisera to CRABP(II) and CRBP (Bucco et al., 1996; Wardlaw et al., 1997).

TABLE 1. Comparison of RA synthesis in normal, immortalized, and malignant breast cells

Cell ID	RA synthesis[1]	CRBP Western[2]	CRABPII Western[2]	P450RAI induction Northern[3]	ER Northern[3]
Normal, first passage					
HMEC1	+++	+	+	0	NT
HMEC2	+++	+	+	0	0
HMEC3	+++	+	+	NT	NT
Immortal, nontumorigenic cell lines					
184B5	++	0	+	0	0
MCF-10F	+++	0	0	+	0
MTSV1.7	+++	0	0	+	0
Cancer cell lines					
T47D	0	0	+	++++	+++
MCF-7	0	0	+	+++	+++
MDA-MB-231	0+	0	0	0	0
ED	+	+	0	0	0
EK	+	+	0	NT	0
MDA-MB-468	+++	+	+	0	0

[1]The ability of each cell population to synthesize RA was evaluated as described in Materials and Methods. Each HMEC culture was analyzed once; therefore, results for cultures established from three different normal tissue specimens are shown. Cell lines were assayed in two (184B5, MDA-MB-231, ED, EK) or 5–10 (MCF-10F, MTSV1.7, T47D, MCF-7, MDA-MB-468) separate occasions. Intraexperimental variation was not routinely monitored because preliminary experiments revealed good agreement between replicates (standard deviations were typically ≤15% of the mean). Interexperimental variation was greater (50% variation was not uncommon) but the ability of a cell line to produce RA or its failure to do so was a consistent observation from experiment to experiment. The results are expressed in a semiquantitative scale: (0) no detectable RA levels; (0+) trace amounts of RA (2 pmol/mg in one assay); (+) low RA levels (5–10 pmol/mg); (++) intermediate RA levels (10–30 pmol/mg); (+++) high RA levels (30–75 pmol/mg). The scores tabulated for cell lines are representative scores.
[2]Summary of results from Figure 2.
[3]Summary of results from Figure 3 and data not shown. NT, not tested.

Northern blot analysis

Total RNA was isolated by using the PureScript RNA isolation kit (Gentra Systems, Minneapolis, MN). Blots containing 20 μg of total RNA/lane were prepared and probed as described (Jing et al., 1996) with, sequentially, full-length hP450RAI cDNA (White et al., 1997), a 316 bp hGAPDH cDNA fragment (Ambion, Austin, TX), and full-length hERα cDNA from pHEGO (Migliaccio et al., 1991). The blots were washed twice at room temperature in 6× SSPE, 0.1% SDS, and once at 65°C in 0.1× SSPE, 0.1% SDS before autoradiography.

Transient transfection

Cells were transfected overnight with 2 μg RAREt-kLUC (Kim et al., 1995), 40 ng pRLSV40 (Promega, Madison, WI), and 8 μg empty vector (pSP65) by using the calcium phosphate method. Following a 6-h interval, the cultures were treated with either all-*trans* retinol (Spectrum Quality Products, Gardena, CA) or all-*trans* RA (Sigma) for 16 h and cell lysates were assayed for luciferase activity by using the dual luciferase assay kit (Promega).

Growth inhibition

Cells were plated at 2,000 cells/well (T47D and MCF-7 cells, 96-well plate, 0.2 ml/well) or 5,000 cells/well (MCF-10F cells, 24-well plate, 1 ml/well). On the following day, fresh growth medium with the indicated concentrations of RA or retinol was added (for these experiments, MCF-10F cells were grown in medium without added epidermal growth factor). T47D and MCF-7 viable cell number was estimated after 6 days of treatment by using the MTT assay as described (Jing et al., 1997). MCF-10F cultures received a medium change after 6 days and viable cell number was assayed at day 8 by using the crystal violet assay as described (Gillies et al., 1986).

RESULTS

Normal and immortal, nontumorigenic breast cells synthesize RA

Three of three independent HMEC cultures established from reduction mammoplasty specimens and analyzed at first passage synthesized RA at high rates (Table 1 and Fig. 1A). No RA was detected in replicate cultures not treated with 2 μM retinol; therefore, the RA detected after retinol treatment reflects the ability of the cultures to carry out de novo RA synthesis. The rate of RA production by HMEC cultures is comparable to or exceeds that seen in other cells actively synthesizing RA (Bucco et al., 1997; Zheng et al., 1999). To our knowledge, this represents the first demonstration of an active RA synthetic pathway in normal breast epithelial cells.

Three of three independent lines of immortalized, nontumorigenic human breast epithelial cells were also capable of synthesizing RA at high rates (Table 1, Fig. 1D). Thus, the ability to synthesize RA is a stable feature of human breast epithelial cells, i.e., is retained following protracted in vitro growth. MTSV1.7, one of the three nontumorigenic cell lines analyzed, is a ductal epithelial cell line (Bartek et al., 1991), thus demonstrating that ductal breast cells are competent in RA synthesis. This is significant in view of the fact that nearly all human breast cancers have a ductal phenotype (Taylor-Papadimitriou et al., 1989).

Experiments in which the RA content of the medium and cells was separately analyzed revealed that, for both normal and nontumorigenic breast cells, most (68–100%) of the RA was recovered from the medium, possibly indicating secretion.

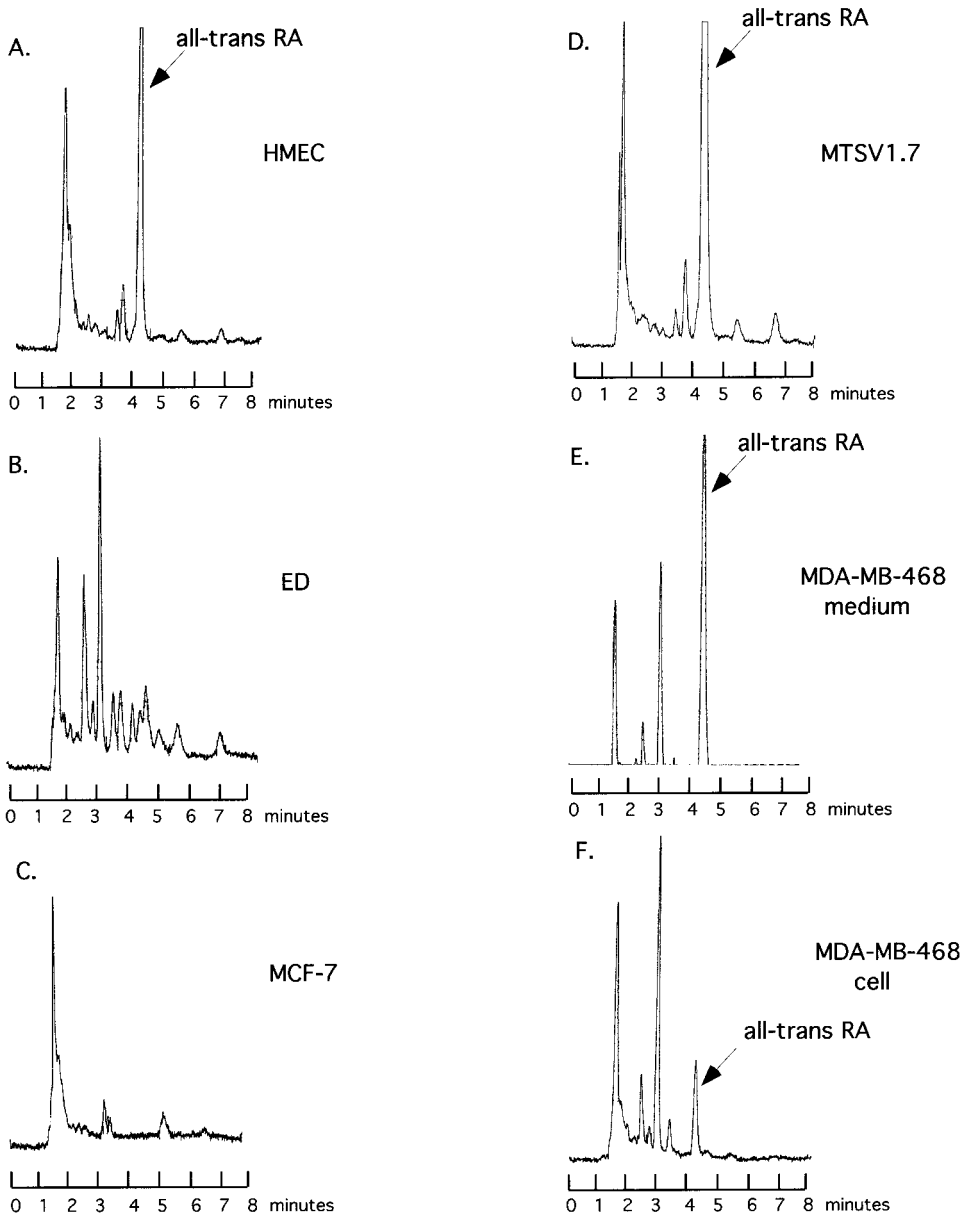

Fig. 1. RA production in breast cells. Cells were treated with 2 μM retinol/8 μM bovine serum albumin (BSA) for 12 h and the amount of all-*trans* RA synthesized during this interval evaluated by HPLC. The chromatogram for one HMEC preparation (**A**) and representative chromatograms for ED cells passage 10 (**B**), MCF-7 cells (**C**), MTSV1.7 cells (**D**), and MDA-MB-468 cells (**E** and **F**) are shown. A through E depict the RA content of culture medium extracts; F depicts intracellular RA content. Arrows point to peaks confirmed to be all-*trans* RA by elution position and absorbance spectrum. See Table 1 and Materials and Methods for details.

RA synthesis is often impaired in human breast cancer cell lines

In contrast to the ability of normal and nontumorigenic breast cells to synthesize RA, five of six human breast cancer cell lines treated with retinol produced no detectable or relatively low levels of RA (Table 1, Fig. 1C). A notable exception was the MDA-MB-468 cell line, which synthesized and secreted RA at high rates (Fig. 1E–F). ED and EK breast cancer cells, which were included in our screen because of their availability at early passage (passage 10–13), exhibited a complex HPLC profile, with multiple nonidentified peaks in addition to a small RA peak (Fig. 1B).

Binding protein expression

In vivo evidence suggests that the coordinate expression of the retinol binding protein receptor, CRBP, retinol, and retinal dehydrogenases is responsible for cellular uptake of circulating retinol and its metabolism to RA at particular sites in the embryo (Bavik et al., 1997; Yamamoto et al., 1998). Both in vivo and in vitro evidence suggest that, via a different system (DE

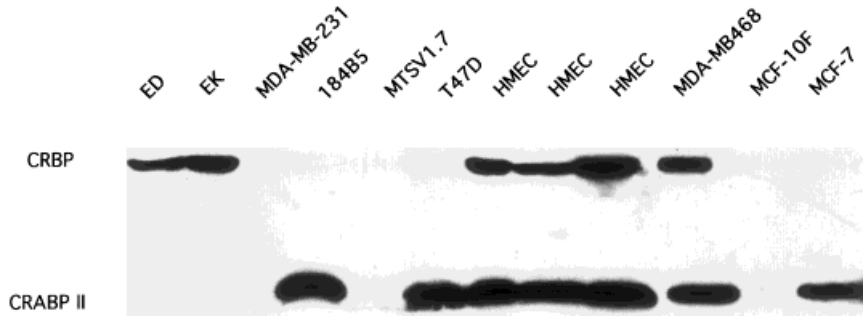

Fig. 2. Retinol and RA binding protein expression. Blots containing 50 μg cytosolic protein/lane were reacted with anti-CRBP or anti-CRABP(II) antibodies and specific reaction products visualized by ECL as described in Materials and Methods. The results shown were confirmed in a second experiment. The data are summarized in Table 1 as either positive or negative binding protein expression.

Ong and B Rexer, unpublished data), CRABP(II) participates in RA production and/or secretion at sites in the female reproductive tract (Bucco et al., 1997; Zheng and Ong, 1998; Zheng et al., 1999). Therefore, we asked whether differential binding protein expression contributed to the observed differences in RA synthesizing ability. As shown in Fig. 2 (and summarized in Table 1), first passage HMEC cultures expressed both CRBP and CRABP(II), which is consistent with binding protein participation in RA synthesis in vivo. However, under the experimental conditions used, and in agreement with earlier reports (Napoli, 1990; Chen et al., 1997), neither binding protein was essential for RA synthesis, as shown, for example, by the results for MTSV1.7 and MCF-10F cells.

P450RAI expression

P450RAI is an RA-inducible RA hydroxylase that has been implicated in RA catabolism (White et al., 1997). Since RA synthesized from retinol might induce P450RAI, which in turn would be expected to lower RA levels, we monitored P450RAI expression in breast cells. Interestingly, one HMEC preparation and 184B5 cells did not express detectable P450RAI, and MTSV1.7 and MCF-10F cells expressed only modest transcript levels following 24-h RA treatment (Fig. 3A); P450RAI is also not induced in MCF-10A cells (White et al., 1997) or in a second HMEC preparation tested by us (data not shown). In agreement with previous reports (Sonneveld et al., 1998), ERα-positive MCF-7 and T47D cells expressed high levels of P450RAI upon RA induction, whereas ERα-negative MDA-MB-231 cells did not; we found that P450RAI was also not induced in the ERα negative cell lines MDA-MB-468 (Fig. 3A) and ED (data not shown). Such pattern of P450RAI expression (summarized in Table 1), taken together with the rapid time-course of its induction (Sonneveld et al., 1998), raised the possibility that RA was synthesized in MCF-7 and T47D cells but was rapidly degraded once P450RAI protein accumulated. However, in the experiment of Fig. 3A, we used 1 μM RA rather than 2 μM retinol, and thus the results were biased toward strong P450RAI induction. Experiments in which T47D cells were treated with 2 μM retinol also revealed enzyme induction, but this effect was at least 10 times weaker (Fig. 3B). Moreover, when T47D and MCF-7 cultures

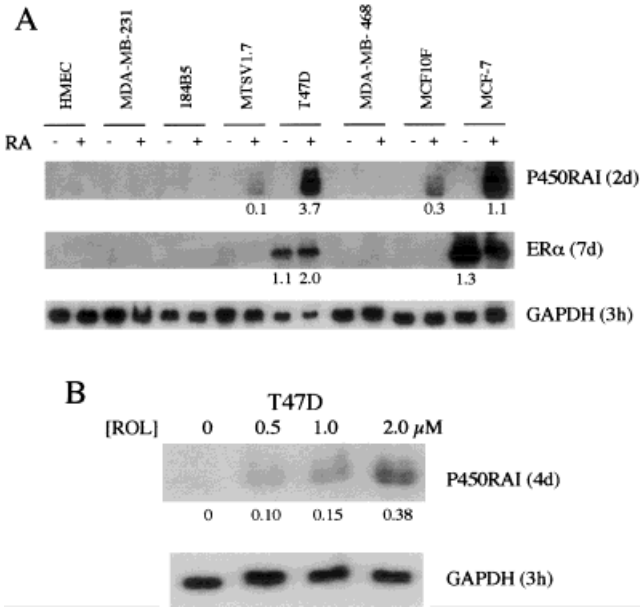

Fig. 3. P450RAI induction. **A:** Cultures of each cell type were treated for 24 h without or with 1 μM all-*trans* RA, total RNA isolated, electrophoresed (20 μg/lane), blotted, and sequentially hybridized to hP450RAI, GAPDH, and ERα cDNA probes as described in Materials and Methods. Autoradiography times are shown in parentheses next to each gene name. The numbers underneath the P450RAI and ERα blots give the ratio of the corresponding mRNA to GAPDH. Note that for some bands exposure times were too long; therefore, the resulting ratios represent an underestimate. **B:** T47D cells were treated with the indicated concentrations of retinol for 24 h, RNA processed as above, and sequentially hybridized to P450RAI and GAPDH cDNA probes. Other details as above.

were treated with 2 μM retinol in the presence of an active concentration (5–10 μM) of the P450RAI inhibitor liarozole (Sonneveld et al., 1998), RA levels remained below detection (data not shown), suggesting that T47D and MCF-7 cells have a primary defect in RA synthesis. Furthermore, MDA-MB-231 cultures do not express P450RAI and yet synthesize very low levels of RA.

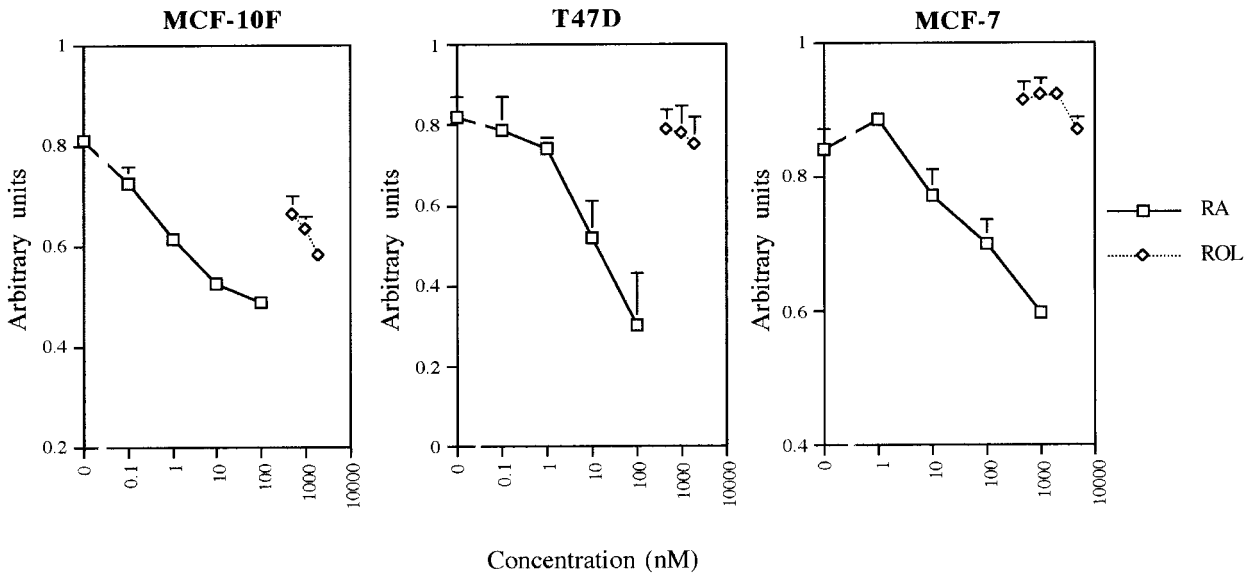

Fig. 4. Growth effects of retinol and RA. Cells were seeded at low density and treated for 6 (T47D, MCF-7) or 8 days (MCF-10F) with the indicated concentrations of RA or retinol. Viable cell number was assayed colorimetrically; the ordinate values are arbitrary values obtained by using a microplate reader (see Materials and Methods for details). The mean of 4–8 replicates and the positive standard deviation or standard error (MCF-7 panel) are shown. Note that the sensitivity to RA varied and that different ordinate scales were used to optimize the display of the results. Similar results to those shown for MCF-10F cells were obtained in a second experiment. In another experiment with MCF-7 cells, we observed 54 and 1% growth inhibition by 1 μM RA and 1 μM retinol, respectively. In the high pharmacological range (10 μM), retinol consistently inhibited the growth of T47D and MCF-7 cells (data not shown).

Retinol bioactivity

The reduced ability of breast cancer cells to convert retinol into RA suggests that the bioactivity of retinol may be compromised in breast cancer. To test this idea, nontumorigenic, RA-producing cells on one hand, and breast cancer cells on the other, were transiently transfected with a RARE reporter and RARE transactivation by 2 μM retinol compared with activation by 10 nM RA. In all cases, 10 nM RA increased RARE transactivation over background levels, and this increase was taken as 100%. We found a small, statistically nonsignificant difference in RARE activation by 2 μM retinol between the two cell groups. Thus, expressed as a percent of activation by 10 nM RA, retinol activation ranged from 76–116%, median 105%, in two RA producer cell lines (MTSV1.7 and MCF-10F) and from 16–79%, median 75%, in three non-RA producer cell lines (MCF-7, T47D, and MDA-MB-231).

As an alternative test of retinol's bioactivity, we compared the ability of retinol and RA to induce cell growth inhibition in RA-sensitive MCF-10F, T47D, and MCF-7 cells. MTSV1.7, MDA-MB-231, MDA-MB-468, and ED cells are not RA-sensitive (Jing et al., 1997 and data not shown) and thus were not studied; 184B5 and EK cells were not tested. As shown in Fig. 4, physiological retinol concentrations (0.5–2 μM) dose-dependently inhibited MCF-10F cell growth but had no comparable effect on T47D or MCF-7 cells, suggesting that there may be a biological correlate to the differences in RA synthesis reported here.

DISCUSSION

The essential finding of this investigation is that normal and immortal, nontumorigenic human breast epithelial cells have an active RA synthesizing machinery. This contrasts with the inability of several human breast cancer cell lines to synthesize RA, at all or at rates that are comparable to those of normal and nontumorigenic cells (Table 1 and Chen et al., 1997). However, as exemplified by MDA-MB-468 cells, even undifferentiated breast cancer cells may retain the ability to synthesize RA at levels comparable to those of nontumorigenic cells.

Our results concerning RA production in MCF-7, T47D, MDA-MB-231, and MDA-MB-468 cells are in good agreement with those of a previous study of retinol metabolism in human breast cells (Chen et al., 1997). On the other hand, our demonstration that normal and immortalized but nontumorigenic breast cells are competent in RA synthesis is not in agreement with that study, which did not detect RA in HMEC 184 or in MCF-10A cells. This discrepancy may be due to the different protocols used: 12-h treatment with 2 μM retinol in our study as opposed to 24-h treatment with 50 nM [^{3}H]-retinol in the study by Chen et al., 1997. Alternatively, since we did not study the exact same cell populations, the difference in our results may stem from subtle differences in cell phenotype. For instance, the HMEC we studied were passaged only once and contained both ductal and basal/myoepithelial cells, whereas HMEC 184 are late passage cells. Perhaps consistent with this suggestion, RA synthesis in two preparations of morphologically senescent HMEC (different from the ones analyzed in Table 1) was in the 0+ to low ++ range, as defined in Table 1. In addition, the MCF-10F cells we studied represent a subpopulation of the MCF-10A cells studied by Chen et al., and culture conditions were somewhat different. The latter may be

a relevant point since we have observed large differences in RA output as a function of medium composition in 184B5 and MCF-10F cells (data not shown). Whatever the explanation for our differing results, our present findings establish that normal and immortalized but nontumorigenic breast cells are competent in RA synthesis. On the other hand, while our data suggest that this is a typical property of these cells, consideration of Chen et al.'s and some of our own data suggest that it is not an invariable property. A recent preliminary report also concluded that HMEC actively synthesizes RA (Hayden et al., 1999).

What impairs RA synthesis in ERα positive (MCF-7, T47D) and ERα negative (MDA-MB-231) breast cancer cells? CRBP and CRABPII participate in one system of RA synthesis under physiological conditions (Bucco et al., 1997; Zheng et al., 1999) and we have shown here that normal breast cells express both binding proteins (Fig. 2); however, as noted earlier, binding protein expression is not a requisite for RA synthesis under the experimental conditions we have used and thus differences in their expression level do not offer an explanation. P450RAI-mediated RA catabolism is also not a likely explanation for the reasons already pointed out: 2 μM retinol does not induce high levels of P450RAI (Fig. 3B) and no RA was detected in T47D and MCF-7 cells treated with retinol in presence of 5–10 μM liarozole. Therefore, we hypothesize that the expression or activity of the RA synthesizing enzymes themselves, which remain to be identified, may be impaired in non-RA producing breast cancer cells. However, we have neither monitored the levels of RA catabolites nor analyzed the expression of P450s known to catabolize retinol (Raner et al., 1996).

Besides T47D and MCF-7, RA synthesis is also defective in another ERα-positive cell line, ZR75-1 (Chen et al., 1997). In contrast, some ERα negative breast cancer cell lines retain at least partial RA synthesizing ability (Table 1 and Chen et al., 1997). This raises the question of whether ERα expression inhibits RA synthesis. ERα expression in the normal breast epithelium is variegated (Petersen et al., 1987), raising the additional question of whether normal ERα positive and normal ERα negative cells differ in their ability to synthesize RA. These and other questions, such as the apparent coamplification of ERα and P450RAI expression in breast cancer (Table 1), are subjects for future study. It should be noted that in contrast to the partial inverse correlation between ERα expression and RA synthesizing ability in breast cancer cells, estrogen administration to prepupertal rats induced the ability to synthesize RA in uterine epithelium 12 h after treatment (Bucco et al., 1997). Estrogen administration to MCF-7 cells did not result in a gain of RA synthesizing ability (data not shown).

We hypothesize that diminished RA synthesis in breast cancer leads to diminished retinol bioactivity. Our RARE transactivation assays did not demonstrate a significant difference in RARE activation by retinol in RA producing versus non-RA producing cell lines. One possible reason for this result is that the non-RA producing cells (T47D, MCF-7, MDA-MB-231) produce 4-oxo-retinol when treated with 2 μM retinol, thus activating the RARE. However, MDA-MB-231 cells are not competent in 4-oxo-retinol synthesis (Chen et al., 1997). Alternatively, then, it is possible that specific or artifactual retinol oxidation generates RA levels that are below the detection limit of our assay (~1 pmol/mg of cell protein) but that are nevertheless sufficient to activate the RARE reporter. In contrast to the RARE experiments, our growth experiments (Fig. 4) provide preliminary support for our hypothesis and encourage further study of what is an often overlooked point in the literature, i.e., cell growth regulation by retinol, the main circulating form of vitamin A. Our results for T47D and MCF-7 cells (poor response to physiological retinol levels) are similar to those reported earlier by Lacroix and Lippman (1980) for MCF-7, Hs578T, and especially ZR75-1 cells.

Several reports have demonstrated that the ability to esterify and store retinol is diminished in cancer relative to the corresponding normal tissue or cells (De Luca et al., 1984; Bhat and Lacroix, 1989; Chen et al., 1997). We have not attempted to analyze retinyl ester formation in our study. However, our results, added to others in the literature (Xu et al., 1995; Pasquali et al., 1996), suggest that decreased retinol conversion to RA is also a common, although obviously not invariable, characteristic of cancer cells. Based on our analysis of cells in vitro, we hypothesize that the normal breast epithelium produces RA, whereas some breast cancer tissues do not. RA treatment has been shown to regulate HMEC cell growth and differentiation (Seewaldt et al., 1997) and we speculate that endogenously synthesized RA may regulate the same functions in vivo. Anti-RA antibodies represent one approach with which to evaluate in situ RA synthesis, but one that is not without shortcomings (Xu et al., 1995). An alternative, long-term approach consists of demonstrating the colocalization in the normal breast epithelium of the protein catalysts and regulators of RA synthesis, and the deregulated expression of one or more of these components in cancer. Conversely, it will be of interest to test whether P450RAI expression is upregulated in ERα positive breast cancer tissue, as suggested by Table 1. Along the first approach, our observation that HMEC express CRBP and CRABP(II), which are both associated with RA synthesis at certain sites in vivo (Bucco et al., 1997; Zheng and Ong, 1998; Zheng et al., 1999), is of interest. We have extended this observation by showing by immunohistochemistry that both binding proteins are expressed in situ in the ductal breast epithelium (data not shown). Further, we have recently demonstrated by in situ hybridization analysis that CRBP expression is downregulated in 24% of human breast cancers (Kuppumbatti et al., 2000).

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