Retinol Metabolism and Lecithin:Retinol Acyltransferase Levels Are Reduced in Cultured Human Prostate Cancer Cells and Tissue Specimens

Xiaojia Guo, Beatrice S. Knudsen, Donna M. Peehl, Alberto Ruiz, Dean Bok, Robert R. Rando, John S. Rhim, David M. Nans, and Lorraine J. Guda

INTRODUCTION

Vitamin A and its natural and synthetic analogues, a group of compounds known as retinoids, have many chemopreventive and chemotherapeutic actions in various experimental models for human cancer (reviewed in Refs. 1–3). In addition, retinoids are currently being used for the treatment of acute promyelocytic leukemia and other types of cancers in humans. The mechanisms by which retinoids regulate the growth and differentiation of cells have not been fully elucidated. The majority of actions of retinoids are thought to be mediated by nuclear receptors, which act as transcription factors. RAR and RXR, retinoid X receptor; HPLC, high-performance liquid chromatography; LRAT, lecithin:retinol acyltransferase; 1α(OH)ase, 25-hydroxyvitamin D-1α- hydroxylase.

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The abbreviations used are: RAR, retinoic acid receptor; RA, retinoic acid; RARE, RA response element; RXR, retinoid X receptor; HPLC, high-performance liquid chromatography; LRAT, lecithin:retinol acyltransferase; 1α(OH)ase, 25-hydroxyvitamin D-1α-hydroxylase.
REDUCED RETINOL METABOLISM AND LRAT IN PROSTATE CANCER

RA-induced growth inhibition was further indicated by a very recent study, performed on human radical prostatectomy tissue specimens, which showed that RAR and RXR mRNAs were selectively lost in both prostate cancer and adjacent, morphologically normal prostatic tissue (43). In addition to the abnormally low levels of RAR and RXRβ in prostate cancers, it was shown that in benign prostatic hyperplasia the concentration of retinol was 2-fold elevated, but in prostate carcinoma the level of RA was five to eight times lower than in normal prostate or in benign prostatic hyperplasia (44). These findings suggest that there are abnormalities in the expression of the enzymes that metabolize retinoids in human prostate cancers.

The pathways for the metabolism of retinoid and RA in various types of cells are complicated and are only beginning to be elucidated at a molecular level (reviewed in Refs. 45 and 46). The cDNA encoding one of the enzymes involved in retinol metabolism, LRAT, was cloned recently (47). We have shown that cultured human carcinoma cells, including those from the head and neck, breast, skin, and kidney, do not esterify retinol to the same extent as normal cultured epithelial cells from these same tissues. These data suggest that the inappropriate cell growth and the loss of normal differentiation responses in these tumor cells may in part result from the lack of a sufficient amount of internal retinol stored as retinyl esters (48–51). In this study, we examined the metabolism of retinol and RA in cultured normal and malignant human prostatic epithelial cells. We show that the cancer cells are not able to metabolize significant amounts of retinol to retinyl esters, and that lack of retinol esterification is correlated with the absence of LRAT protein expression. We also show that LRAT protein is not expressed in prostatic carcinoma cells in tissue specimens from human patients.

MATERIALS AND METHODS

Materials. Radiolaabeled retinol (all-trans [11,12-^3H]; specific activity, 27–47 Ci/mmol) was purchased from New England Nuclear/Dupont (Boston, MA). All other chemicals used, unless specified, were purchased from Sigma Chemical Co.-Aldrich (St. Louis, MO).

Cells and Culture Conditions. The origins and properties of the cell strains and lines used have been described previously (Table 1). One normal human prostatic epithelial cell strain (PrEC) was obtained from Clonetics Corp. (Walkersville, MD) and was cultured according to the manufacturer’s instructions. The other normal cell strain (E-PZ-10) was derived in the laboratory of Dr. Peehl from histologically normal tissue of the peripheral zone of a radical prostatectomy specimen (52). Culture methods were as described previously (52). Epithelial cell strains were also derived from adenocarcinomas of the prostate of Gleason grade 3/3 (E-CA-11), 3/4 (E-CA-21), 4/3 (E-CA-91), and 4/4 (E-CA-37). The PRNS-1–1 line and pRNS-1–1/ras cell lines were derived and cultured as described previously (42). The LNCaP, DU 145, Tsu-Pr1, PPC-1, PJ-1, and PC-3 cells were maintained in a consensus medium consisting of a mixture of DMEM and Ham’s F-12 medium (1:1) supplemented with 5% FCS, 0.4 μg/ml hydrocortisone, 10 μg/ml epidermal growth factor, and 5 μg/ml insulin (53). Normal human mammary epithelial cells were from Clonetics and were cultured as described previously (50). For radiolabeling studies, Northern and Western analyses of all of the cell strains and lines and a consensus medium consisting of DMEM plus 5% fetal bovine serum were used.

[^3H]Retinol Radiolabeling, Extraction of Retinoids, and HPLC. Radio-labeled and retinoid extractions were as described previously (49). Nonradiolabeled retinoid standards were added to the samples before extraction. Briefly, 50 μl of acetone:toluene:butanol (50:50:5, v/v) and 0.1% butylated hydroxytoluene were added to 0.5 ml of cells or medium samples. The mixtures were vortexed thoroughly for 30 s. After addition of 500 μl of a saturated (1.3 kg/l) K2HPO4 solution and thorough mixing, the samples were centrifuged for 10 min at 3000 × g. The upper organic layer was collected and transferred to an injector vial for automated HPLC analysis.

The HPLC analysis was performed using a Waters Millennium system (Waters Corp., Milford, MA) to separate the various retinoids. Samples were applied to an analytical 5-μm reverse-phase C18 column (Vydac, Hesperia, CA) at a flow rate of 1.5 ml/min, as described (48–51).

Retinoids were identified by HPLC based on at least two criteria: an exact match of the retention times of unknown peaks with those of authentic retinoid standards and identical UV spectra (220–400 nm) of unknowns against spectra from authentic retinoid standards during HPLC by the use of the photodiode array detector. RA was also identified by the shift of the retention time of the methylated RA derivative (48).

Western Analysis. This procedure was carried out as described previously (47, 50, 51). Briefly, polyclonal antiserum were generated to a mixture of two different LRAT peptides in rabbits. Total cell protein was used, and Western blot analysis on nitrocellulose filters was performed using antisera diluted to 1:500. As a control, the antibody was preincubated with the peptide. Sections were stained using the TechMat 500 machine as specified by the manufacturer (Verhave, Inc.). Secondary antibodies were horseradish peroxidase conjugated (1:1000 dilution), and diaminobenzidine was used as a substrate. Several specimens from different patients were examined. Slides were analyzed by a pathologist (B. S. K.).

RESULTS

Metabolism of[^3H]Retinol in Cultured Normal and Malignant Human Prostatic Epithelial Cells. We first examined[^3H]retinol metabolism in cultured normal and malignant human prostatic epithelial cells to determine whether differences in retinol metabolism are present. Cells were cultured in the presence of 50 nM[^3H]retinol for various times (22 h is shown in Fig. 1). Cells and medium were then harvested, retinoids were extracted, and the metabolites of[^3H]retinol were analyzed by HPLC. Nonradiolabeled retinoid standards were added to each sample to allow the identification of many of the radiolabeled retinoids. The HPLC tracings of[^3H]retinol and its metabolites from two normal human prostatic epithelial cell strains, PrEC and E-PZ-10, and from six prostate cancer cell lines are shown (Fig. 1). The normal cell strains esterified almost all of 50 nM[^3H]retinol, whereas the ability to esterify[^3H]retinol was greatly impaired in all prostate cancer lines (Fig. 1).

[^3H]RA was synthesized from[^3H]retinol by the PC-3 prostate cancer line, whereas only trace amounts of[^3H]RA were produced by the LNCaP, DU 145, and Tsu-Pr1 tumor lines. The normal epithelial cell strains did not synthesize detectable amounts of[^3H]RA from[^3H]retinol (Fig. 1).

Table 1. Cell strains and lines

<table>
<thead>
<tr>
<th>Cell strains and lines</th>
<th>Description</th>
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<tbody>
<tr>
<td>PrEC</td>
<td>Normal human prostate epithelial cell strain</td>
</tr>
<tr>
<td>E-PZ-10</td>
<td>Normal human prostate epithelial cell strain</td>
</tr>
<tr>
<td>LNCaP</td>
<td>Androgen-sensitive human prostate cancer line</td>
</tr>
<tr>
<td>PC-3^a</td>
<td>Androgen-independent human prostate cancer line</td>
</tr>
<tr>
<td>DU 145</td>
<td>Androgen-independent human prostate cancer line</td>
</tr>
<tr>
<td>TSU-Pr1</td>
<td>Androgen-independent human prostate cancer line</td>
</tr>
<tr>
<td>PJ-1</td>
<td>Androgen-independent human prostate cancer line</td>
</tr>
<tr>
<td>PPC-1^a</td>
<td>Androgen-independent human prostate cancer line</td>
</tr>
<tr>
<td>pRNS-1-1</td>
<td>Normal human prostate epithelial cells immortalized with SV40</td>
</tr>
<tr>
<td>pRNS-1-1/ras</td>
<td>Normal human prostate epithelial cells immortalized with SV40 and stably transduced with activated ras; this line forms tumors in nude mice</td>
</tr>
<tr>
<td>E-CA-11</td>
<td>Culture of human prostate cancer primary cells from patient 1; Gleason grade 3/3</td>
</tr>
<tr>
<td>E-CA-21</td>
<td>Culture of human prostate cancer primary cells from patient 2; Gleason grade 3/4</td>
</tr>
<tr>
<td>E-CA-91</td>
<td>Culture of human prostate cancer primary cells from patient 3; Gleason grade 3/3</td>
</tr>
<tr>
<td>E-CA-37</td>
<td>Culture of human prostate cancer primary cells from patient 4; Gleason grade 4/4</td>
</tr>
</tbody>
</table>

^a These two cell lines are of the same origin (53).

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The calculation of the total amount of [3H]retinol remaining over time is an indicator of the amount of retinol metabolized into all types of metabolites; all of the [3H]retinol remaining, including both the [3H]retinol still in the medium and the intracellular [3H]retinol, was measured. The time course of [1H]retinol metabolism is shown in Fig. 2. The normal prostatic epithelial cell strain PrEC metabolized ~75% of the 50 nM [3H]retinol within 4 h, whereas the two prostate carcinoma lines, LNCaP and PC-3, metabolized <5% of the [3H]retinol in the 24-h time period. Two additional cell lines were tested in this assay, pRNS-1-1 and pRNS-1-1/ras. pRNS-1-1 is a line of normal human prostatic epithelial cells immortalized with SV40, and the tumorogenic pRNS-1-1/ras line was created by stable transfection with activated ras. These two cell lines metabolized a modest amount of [3H]retinol over the 24-h time period, approximately 20–25% of the total [3H]retinol (Fig. 2). Analysis of LRAT Protein Expression. Because of the large reduction in [3H]retinol esterification observed in the prostatic carcinoma cells as compared with the normal prostatic epithelial cells, we examined the expression of the LRAT protein, which is responsible for retinol esterification in many cell types, by Western analysis using a polyclonal antibody against human LRAT (47). LRAT protein with a molecular mass of approximately 62–65 kDa was observed in the

Fig. 1 Metabolism of [3H]retinol in normal human prostate epithelial cell strains and in human prostate carcinoma lines. The cells were cultured in the presence of 50 nM [3H]retinol for 22 h. Cells and one-fourth medium were then harvested, and retinoids were extracted and separated by reverse-phase HPLC analysis. One representative HPLC tracing for each cell strain or line is shown. Only the intracellular retinoids are shown. Nonradioabeled retinoids were included with each sample as standards to determine the elution times of the various retinoids. The data for each sample are plotted as [3H] cpm versus time per 10^6 cells. The peaks that correspond to [3H]RA (RA), [3H]retinol (ROH), and the various [3H]retinyl esters (RE) are at 18.5, 29.5, and 47–58 min, respectively. This experiment was performed three times with very similar results.

Related data from other prostatic cancer cell lines are shown in Fig. 3B. None of the six tumor cell lines tested exhibited significant esterification of [3H]retinol to the [3H]retinyl esters. The time course of the formation of the intracellular [3H]retinyl esters is shown in Fig. 3C. It can be seen that formation of the intracellular [3H]retinyl esters in the normal prostatic epithelial cells was rapid, with the majority of the retinyl esters synthesized from retinol in the first 4 h after the addition of 50 nM [3H]retinol to the medium. In contrast, the pRNS-1-1, pRNS-1-1/ras, LNCaP, and PC-3 tumor cell lines exhibited much less synthesis of [3H]retinyl esters even at 22 h after [3H]retinol addition (Fig. 3C). These results from the prostate cancer cells (Figs. 1–3) are similar to those that we reported previously for human carcinoma cells from the oral cavity, breast, and kidney (48–51).

It was shown previously that pretreatment with RA could increase retinol esterification in some cell types. To ascertain whether this would also be true in prostate cells, normal and malignant cells were cultured in 1 µM RA for 48 h. The medium was then changed, and [3H]retinol was added to the medium. Culture of the cells for 48 h in the presence of 1 µM RA before the treatment with [3H]retinol increased the levels of intracellular retinyl esters in all of the cells except for LNCaP and PC-3 by approximately 15–30% (Fig. 3A).

It was apparent from the tracings in Fig. 1 that much of the [3H]retinol was esterified by the normal prostatic cells. The intracellular concentrations of the [3H]retinyl esters synthesized from [3H]retinol are shown in Fig. 3. Only the normal cell strains exhibited high intracellular levels of these [3H]retinyl esters. The two cancer lines LNCaP and PC-3 exhibited almost no intracellular [3H]retinyl esters (Fig. 3A). The levels of [3H]retinyl esters in the pRNS-1-1 and pRNS-1-1/ras lines were also much lower than those in the normal epithelial cell strains (Fig. 3A). Primary epithelial cell strains isolated from adenocarcinomas of Gleason grades 3 and/or 4 (see Table 1) were analyzed similarly. All of these cancer cell strains also exhibited lower levels of [3H]retinyl esters as compared with the levels in the normal cell strains (Fig. 3A).

Fig. 2 Metabolism of [3H]retinol in prostatic epithelial cells over time. Cells were cultured as in Fig. 1 and analyzed by HPLC. Shown is the total (intracellular plus in the medium) [3H]retinol remaining at various times (4, 15, and 24 h) after addition of 50 nM [3H]retinol to the cells at time zero, calculated as described previously (48, 49).
PrEC protein extract but not in extracts from the prostate cancer lines (Fig. 4A). In addition, the level of LRAT protein was greatly reduced in the pRNS-1-1 and pRNS-1-1/ras lines relative to the normal PrEC cells (Fig. 4B). These protein data are consistent with the data shown in Fig. 1 indicating that the PrEC cells esterify much more retinol than the prostate cancer cells. These data strongly suggest that LRAT protein is expressed at much lower levels in the carcinoma cells as compared with the normal prostate epithelial cells.

The Metabolism of $[^{3}H]$RA in Normal versus Malignant Prostate Epithelial Cells. In this series of experiments, normal and cancer cells were treated with 50 nM $[^{3}H]$RA for various times, followed by cell harvest and retinoid extraction. The metabolites of $[^{3}H]$RA were then examined by HPLC (Fig. 5). A low rate of $[^{3}H]$RA metabolism was observed in the majority of the cancer cell lines, although the DU 145 and PJ-1 cancer lines metabolized almost all of the $[^{3}H]$RA over a 20-h culture period (Fig. 5). Quantitation of $[^{3}H]$RA metabolism in these various lines indicated that ~95% of the 50 nM $[^{3}H]$RA was metabolized in 20 h by the DU 145 and PJ-1 cell lines, whereas the LNCaP and PC-3 cell lines metabolized almost none of the $[^{3}H]$RA during this time period (data not shown). The normal cell strains metabolized only a moderate amount of $[^{3}H]$RA (data not shown).

The ability to metabolize $[^{3}H]$RA to more polar metabolites in the DU 145 line correlated with the expression of CYP26 (RA hydroxylase) transcripts (data not shown). The cytochrome P-450 enzyme CYP26 uses RA as a substrate (54–59). Only the DU 145 cells expressed significant levels of CYP26 mRNA, and this was seen only after the cells were cultured in the presence of 1 μM RA for 48 h. Although the normal cells and PJ-1 cells metabolized some $[^{3}H]$RA, these cells did not express CYP26 mRNA (data not shown), suggesting that other enzymes may carry out RA metabolism in these cells.

Cell Growth Inhibition by Retinooids. We compared the growth inhibition by RA and 4-oxoretinoic acid in the prostate cancer cell lines DU 145, PC-3, and LNCaP to ascertain whether growth inhibi-
tion correlated with the reduced ability to metabolize RA, because this would result in higher internal RA levels. If this were the case, the DU 145 line would be less growth inhibited by RA than the PC-3 and LNCaP tumor lines. However, no correlation was found. All three of the cell lines were not growth inhibited by either RA or 4-oxoretinoic acid (Fig. 6). Thus, although a correlation between RA metabolism and RA growth inhibition has been observed in some tumor lines (60–62), there was no correlation between the ability of the cells to metabolize RA and the inhibition of the growth of these cells by all-trans RA and 4-oxoretinoic acid.

**LRAT Expression in Human Prostate Tumor Specimens.** Prostate cancer tissue specimens and benign human prostatic tissues were procured from the Pathology Department, Weill Medical College of Cornell University. The expression of LRAT was analyzed in benign prostate epithelium and in prostate cancer by immunohistochemical staining of five separate radical prostatectomy specimens. In all cases, LRAT was highly expressed in basal prostate epithelial cells, whereas secretory cells stained to a lesser extent (Fig. 7A). Stromal cells were also negative. The preimmune serum from the same rabbit did not react with basal cells (Fig. 7B). In the six prostate cancer cases with Gleason grades 3 or 4, the tumor cells did not express LRAT (one sample; Fig. 7A, arrows). Thus, these *in vivo* findings corroborate the results from cell culture studies. The primary prostate epithelial cell cultures consist of basal and intermediate/transiently proliferating cells and show high LRAT expression (Fig. 4). In contrast, primary cultures derived from tumor tissue, ras-transformed primary cells, and prostate cancer cell lines are negative for LRAT expression. Taken together, these data strongly suggest that prostate cancer cells do not express LRAT and that the culture system can be used for further studies of the regulation of LRAT expression.

**DISCUSSION**

We have demonstrated that human prostatic carcinoma cells exhibit a greatly reduced ability to metabolize [3H]retinol to [3H]retinyl esters...
relative to normal prostatic epithelial cells (Fig. 1). These data extend our previous research, which has shown that the ability to metabolize [\(^{3}H\)]retinol to [\(^{3}H\)]retinyl esters is greatly decreased in carcinoma cells from the breast (48), the oral cavity and skin (49, 50), and the kidney (51). LRAT is the enzyme responsible for this metabolism (Fig. 8).

We also demonstrate that prostatic cancer cells express little or no LRAT protein as compared with normal prostatic epithelial cells (Figs. 4 and 7). We do not yet know the mechanism by which the expression of the LRAT protein is altered in cancer cells versus normal epithelial cells. However, our results to date strongly suggest that a major alteration in LRAT transcripts and the lack of expression of LRAT protein are common features of cultured human carcinoma cells (48–51). In skin, the other tissue system in which we have examined LRAT expression by immunocytochemistry, LRAT is expressed essentially in a gradient, with most dense expression in the basal layer and least dense expression in the most superficial, upper layer of the skin (49, 50).

It is interesting to note that expression of the gene 1α(OH)ase is also significantly decreased in both primary cultures of prostatic cancer cells and prostate cancer cell lines compared with normal prostatic epithelial cells (63). The enzyme 1α(OH)ase converts 25-hydroxyvitamin D\(_3\) to the active metabolite 1α,25-hydroxyvitamin D\(_3\). Extensive studies have demonstrated antiproliferative and prodifferentiation activity of 1α,25-hydroxyvitamin D\(_3\) on prostatic epithelial cells (64). Loss of activity of two enzymes, LRAT and 1α(OH)ase, relevant to the synthesis of two potent antiproliferative and prodifferentiation factors would be biologically significant in the development of prostatic cancer.

Tumor progression occurs in most types of cancers, including prostate cancer (65–68). The increasing tumorigenicity of the cells is associated with increased numbers of mutations and chromosomal instability (reviewed in Refs. 65–68). Analyses using established cancer cell lines are not useful for determining whether the loss of LRAT protein expression is an early or late event in tumor progression, because all of the tumor lines were established from late-stage cancers. However, the results from our analyses of primary cultures derived from cancers of Gleason grade 3 (well-differentiated) and grade 4 (less differentiated) suggest that loss of LRAT may be an early event because even the cell strain from grade 3/3 cancer did not express LRAT. The pattern of expression of LRAT in the pRNS-1-1 cell line versus the pRNS-1-1/ras cell line also supports the possibility that the loss of LRAT protein expression is an early event in the tumorigenesis pathway. As shown in Fig. 4B, the pRNS-1-1 line, immortalized with the SV40 T antigen, exhibited a low level of expression of the LRAT protein, whereas the tumorigenic pRNS-1-1/ras line did not exhibit detectable LRAT expression. Consistent with this, these two cell lines exhibited much lower levels of retinol esterification than those observed in normal prostatic epithelial cell strains (Fig. 3A). Intriguingly, the pRNS-1-1/ras cells were unresponsive to the growth-inhibitory signals of RA, whereas the pRNS-1-1 cells were sensitive to RA growth inhibition (42).

Normal human prostate epithelia consist of two cell layers, the basal layer and the luminal or secretory layer. The basal cells have proliferative potential and are classically identified by markers such as keratins 5 and 14. Differentiated secretory cells express keratins 8 and 18, prostate-specific antigen, and androgen receptors (69). Malignant prostatic epithelium does not contain basal cells. Given that LRAT expression was observed primarily in the basal cells of the normal prostate epithelium in our immunohistochemical studies, the lack of expression of LRAT in cancer may result from the absence of basal cells in cancer. However, the cell culture data suggest that this explanation is too simplistic. The cultured normal prostate epithelial cells correspond to the basal cells and/or “transiently proliferating/amplifying” cell population that has both a shorter life span and the ability to proliferate (69, 70). Primary cultures of both normal and cancer-derived cells express markers of basal as well as secretory cells, with no apparent differences between the normal and malignant cells (52, 71). Therefore, the differential expression of LRAT between the primary normal and cancer-derived cultures cannot be correlated with a basal versus a secretory phenotype. Although “normal” cells immortalized by SV40 maintain some LRAT expression, upon transformation by the ras oncogene, LRAT protein expression is lost. Assuming that introduction of oncogenic ras does not cause differentiation but increases oncogenicity, the results are consistent with the loss of LRAT protein as a result of malignant transformation. The decrease in LRAT expression in epithelial cells derived from prostate cancer tissue compared with cells obtained from areas of nonmalignant prostate supports the argument that LRAT is down-regulated as a result of oncogenic transformation.

There is a large amount of data for the prostate and other types of epithelia that retinoids are required for normal differentiation (33, 72–74). Expression of the LRAT enzyme would allow basal cells to accumulate and store large amounts of retinyl esters, later using hydrolyase enzymes to convert the retinyl esters to more active retinoids such as RA upon differentiation into luminal cells. When the


41. Lee, M. S., Garkavenko, E., Yun, J. S., Weijermans, P. C., Peehl, D. M., Chen, L. S., and Bhim, J. S. Characterization of adult human prostatic epithelial cells immortal-


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