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Vitamin A Metabolism is Impaired in Human Ovarian Cancer

Stephen J. Williams¹, Dusica Cvetkovic, and Thomas C. Hamilton

Ovarian Cancer Program, Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, PA, USA

Abstract

Objectives—We have previously reported that loss in expression of a protein considered critical for vitamin A homeostasis, cellular retinol-binding protein 1 (CRBP1), is an early event in ovarian carcinogenesis. The aim of the present study was to determine if loss of vitamin A metabolism also occurs early in ovarian oncogenesis.

Methods—We assessed CRBP1 expression by immunohistochemistry in ovaries prophylactically removed from women with a genetic risk for ovarian cancer. Furthermore, we investigated the ability of normal, immortalized but nontumorigenic, and tumorigenic human ovarian epithelial cells to synthesize retinoic acid and retinaldehyde when challenged with a physiological dose of retinol, and determined expression levels of the retinoid-related genes, RAR α , RXR α , CRABP1, CRABP2, RALDH1 and RALDH2 in these cells.

Results—Immunohistochemistry revealed loss of CRBP1 expression in potentially preneoplastic lesions in prophylactic oophorectomies. HPLC analysis of vitamin A metabolism showed production of retinoic acid in four independent, normal human ovarian surface epithelial (HOSE) cell culture upon exposure to retinol. However, only one of two SV40-immortalized HOSE cell lines made RA, while none of the ovarian carcinoma cell lines produced detectable RA due to complete loss of RALDH2.

Conclusions—The impaired conversion of retinol to RA in ovarian cancer cells, and decreased CRBP1 protein expression in prophylactic oophorectomies support our hypothesis that concomitant losses of vitamin A metabolism and CRBP1 expression contribute to ovarian oncogenesis.

Keywords

Vitamin A; Cellular retinol-binding protein 1; Cancer; Ovarian

Introduction

Retinoids have been shown to be important in oncogenesis for many tissues [1], and alterations in vitamin A and retinoid homeostasis are found in many tumors, including leukemia, breast, oral, prostate and carcinoma of the cervix [1–4]. Mira-y-Lopez and colleagues reported

1Corresponding author: Stephen J. Williams, 333 Cottman Avenue W310, Philadelphia, PA 19111, Phone 215-728-3679, Fax 215-728-2741, E-mail address: SJ_Williams@fccc.edu.

¹Abbreviations: CRBP1, cellular retinol-binding protein 1; HOSE, human ovarian surface epithelium; RA, retinoic acid; FFPE, formalin-fixed paraffin-embedded; PBS, phosphate-buffered saline; 4HPR, phenylretinamide; IHC, immunohistochemistry; RAL, retinaldehyde

Conflict of interest statement

The authors declare there are no conflicts of interest.

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impaired conversion of retinol into retinoic acid (RA) in breast cancer cell lines compared to normal mammary epithelium [5]. Altered retinol metabolism was also shown in prostate carcinoma relative to normal epithelia [6,7]. Defects in genes associated with retinol homeostasis, notably cellular retinol binding protein 1 (CRBP1), a key component in retinoid homeostasis [8–10], have been linked to the oncogenic process. Jing and co-workers reported constitutively lower expression of CRBP1 in breast cancer cell lines compared to normal breast tissues [11], and Kuppumbatti et al. showed lack of CRBP1 expression in a subset of breast cancers [12]. Loss of CRBP1 expression has been shown in nasopharyngeal [13], esophageal [14], gastric, colorectal [15], liver [16], prostate [17], breast [12] and renal cancer [18], as well as leukemic cells [19] and is often the result of hypermethylation of CpG islands in the CRBP1 promoter [15], a process known to be inhibited by RA [20]. In fact, cells from tumors with low CRBP1 expression have complete loss of RA production [5] [21].

Clinical studies have resulted in controversy as to the role of vitamin A in ovarian oncogenesis [22–26], including a chemopreventive fenretinide trial [27,28]. We have previously developed an in vitro rat model of ovarian cancer [29,30] and discovered consistent loss of expression of genes involved in vitamin A homeostasis, including CRBP1 [31]. These findings were translated to human ovarian cancer where CRBP1 expression was lost in tumor cell lines and in microdissected tumor tissue specimens [32].

Based on the aforementioned studies, it appears that loss of CRBP1 and retinol metabolism is a frequent feature in many malignancies. Therefore, in this report we test the hypothesis that the loss of CRBP1 results in loss of vitamin A metabolism.

Materials and methods

Ovarian tissue specimens and immunohistochemistry

All experimental protocols involving usage of normal and tumor cells and tissues were examined and approved by the FCCC Institutional Review Board. Ovarian tissue specimens from women at perceived increased risk of ovarian cancer who had surgical prophylactic oophorectomies (n=24) and were referred through Family Risk Assessment Program (FRAP), and control women considered at background risk for ovarian cancer, who had their ovaries removed for diseases unrelated to ovarian cancer (n=4), were collected and examined for CRBP1 immunostaining. FRAP criteria for prophylactic surgery was either the identification of BRCA1/2 mutations or a family history of predisposition to breast and/or ovarian cancer (multiple cases of early onset breast cancer, ovarian cancer, breast and ovarian cancer in the same woman, bilateral breast cancer, male breast cancer and Ashkenazi Jewish heritage). The risk of ovarian cancer for women in this study has not been calculated using any of the existing quantitative models. Follow up information on these patients is available.

Tissue localization of CRBP1 protein was performed using an affinity-purified, polyclonal rabbit anti-human CRBP1 antibody [32–34]. Tissue sections (5-µm) were cut from paraffin blocks, deparaffinized and hydrated through xylenes and graded alcohol series. After rinsing in phosphate-buffered saline (PBS) solution and blocking in 3% hydrogen peroxide for 20 minutes, the sections were washed in PBS, and nonspecific binding was blocked for 30 minutes in normal 10% goat serum (Biogenex, San Ramon, CA). This was followed by 15 minutes of avidin block and 15 minutes of biotin block. No antigen retrieval method was applied before immunostaining. Tissue sections were incubated with 0.4 µg/ml of the CRBP1 antibody overnight at 4°C. The negative control sections were treated identically to all other sections except that PBS was used in place of the primary antibody. The antigen was detected with the Super Sensitive Detection Kit (Biogenex). The sections were incubated for 30 minutes with the secondary biotinylated antibody (goat antirabbit IgG); 30 minutes with streptavidin-HRP reagent, finally with chromogen 3,3'-diamino benzidine (DAB) for 4 minutes, and washed with

deionized water. Gill's hematoxylin was used for counterstaining. Two independent observers who were blinded to the pathological status carried out the analysis of CRBP1 staining in ovarian specimens. If 10% of cells stained positive (brown) for CRBP1, we recorded it as positive.

Tissue localization of aldehyde dehydrogenase family 1 member A2 (ALDH1A2) protein was performed using an affinity-purified goat polyclonal anti-human ALDH1A2 antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) at 1:50 dilution, following the above protocol.

Cell culture

Cultures of normal human ovarian surface epithelial (HOSE) cells used here (HOSE1, HOSE2, HOSE3 and HOSE4) were established by scraping the surface of grossly and microscopically confirmed normal ovaries, as described previously [35,36]. These specimens were obtained from control women who had their ovaries removed for reasons unrelated to ovarian cancer (lymphoma, endometrial cancer and two with benign diseases not involving the ovary) and were referred through FCCC Family Risk Assessment Program. Their BRCA1/2 mutation status is unknown. These women are considered at background risk for ovarian cancer. HOSE113 and HOSE118 cells were transfected with SV40 large T antigen expression vector to extend their lifespan in culture, though remaining nontumorigenic, as described previously [37]. The malignant human ovarian cell lines (OVCAR3, OVCAR10, A1847, A2780, SKOV3, PEO1) were previously established [38].

To determine the growth effects of all-trans retinoic acid (atRA) and retinol (ROH) on normal and transformed HOSE, 2000 HOSE cells or 1000 ovarian carcinoma cells per well were plated in 96-well plate. Cells were treated, in triplicate, with increasing concentrations of atRA or ROH for 12 days. Analysis of the kinetics of RA-induced growth inhibition revealed that, by day 6 of 10 μ M atRA exposure, $48 \pm 10\%$ OVCAR3 cells were still viable ($p \leq 0.008$, $n=3$) while neither HOSE nor HOSE118 were growth inhibited. Therefore, considering the growth kinetics of HOSE cells in culture, the retinoid incubations were carried out for 12 days. Cell proliferation was assayed using the CellTiter Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI). All compounds were dissolved in ethanol. No effect of ethanol was seen in controls.

RNA extraction and semi-quantitative PCR

Total RNA was extracted from cultured ovarian epithelial cells by TRIzol (Invitrogen, Carlsbad, CA). One μ g of RNA, dissolved in DEPC-treated water, was reverse-transcribed (RT) in a 20 μ l reaction containing 25 μ g/ml oligo (dT)₁₅ primer (Promega), 100 μ M dTP mix, 1 $\times$ RT buffer, and 200 units of M-MLV Reverse Transcriptase (GenHunter, Nashville, TN). The reaction conditions were 5 min at 65°C, 1 h at 37°C, and 10 min at 70°C. A 4 μ l aliquot was used in a 20 μ l PCR reaction containing 1 $\times$ PCR buffer with 1.5 mM MgCl₂ (Roche Applied Science, Indianapolis, IN), 1 μ M PCR primers and 1.5 units of Amplitaq® DNA Polymerase [39]. CRABP1 PCR primers were described in Uchida et al. [40], and RALDH1 and RALDH2 PCR primers in Rexer et al. [41]. Primers were constructed spanning introns as to limit any genomic contamination. The forward CRABP2 primer was 5'-CCC AAC TTC TCT GGC AAC TGG-3' and the reverse 5'-CTC TGC GAC GTA GAC CCT GGT-3'. The forward RAR α primer was 5'-GTC TTT GCC TTC GCC AAC CAG-3' and the reverse 5'-GCC CTC TGA GTT CTC CAA CA-3'. The forward RXR α primer was 5'-ATC AGC AAA GAC CTC AGC CGC-3' and the reverse 5'-AGG CTC TGG GTG AAC AAC GCT G-3'. The forward β -actin primer was 5'-GGC GGC AAC ACC ATG TAC CCT and the reverse 5'-AGG GGC CGG ACT CGT CAT ACT-3'. PCR reaction conditions were as follows: samples denatured for 1 min at 95° followed by 33 cycles of 30 s at 95°C, 45 s at 60°C, and 1 min extension at 72°C (CRABP1 and CRABP2), 32 cycles of 30 s at 95°C, 30 s at 50°C, 1.5 min

extension at 72°C (RALDH1 and RALDH2) and 18 cycles of 30 s at 95°C, 45 s at 60°C, and 1 min extension at 72°C (β -actin).

Real-time RT-PCR

RNA was isolated from a panel of ovarian cells and reverse-transcribed as described above. Quantitative real-time PCR analysis was performed in the SmartCycler System (Cepheid, Sunnyville, CA) to simultaneously amplify CRBP1 and the housekeeping gene GAPDH. Real-time PCR products were detected from 25 ng of cell cDNA as described previously [32]. We chose to compare CRBP1 mRNA expression in HOSE2, HOSE3, HOSE4, HOSE118, A2780, SKOV3, OVCAR3 and OVCAR10 cells to HOSE1 cell expression. Threshold cycle (C_t) was defined as the cycle number when amplification of a specific PCR product can be detected, as calculated by the SmartCycler software. Differences in gene expression were calculated using the following formula:

$$\text{Fold Difference} = 2^{(\text{HOSE } C_{t\text{CRBP1}} - \text{GAPDH} - \text{QUERY } C_{t\text{CRBP1}} + \text{GAPDH})}$$

HPLC analysis of vitamin A metabolites

HPLC separation coupled with electrochemical detection was based on the method by Sakhi et al. [42], with minor modifications. Retinoids 9-cis-RA, 13-cis-RA, atRA and all-trans retinol were separated on a 250 $\times$ 4.6 mm Suplex pKb-100 reverse phase column packed with 5 μ m silica (Supelco, Bellefonte, PA), with a 20 $\times$ 4.0 mm Supelguard column, with a mobile phase of acetonitrile:methanol:2% ammonium acetate:glacial acetic acid (79:2:16:3, v/v).

Electrochemical detection was performed using a Coulochem II Detector (ESA Biosciences, Inc., Chelmsford, MA). The analytic cell consisted of two cells in series, a conditioning cell at 450 mV and the detection cell at 750 mV. Peak area was calculated using Agilent ChemStation software (Agilent, Santa Clara, CA). The detection limit for all retinoids was at least 300 pg in 100 μ l injection volume. For analysis of retinoid metabolism, 4×10^5 cells, seeded in triplicate in 6-well plates, were incubated for 16 h with either 2 μ M all-trans retinol (Sigma, St. Louis, MO), or 0.1% ethanol (as control) in 2 ml of complete medium. Human plasma contains 2–2.5 μ M all-trans retinol under physiological conditions [43]. For extraction of retinoids, 2 ml of the incubation medium was acidified with 200 μ l of 2.5 M ammonium acetate (pH 4.0), 150 ng of internal standard (IS) 4-hydroxy-phenylretinamide (4HPR) (Sigma) was added, and deproteinated with 3.3 ml of acetonitrile. Samples were subject to solid phase extraction using a Supelcosil LC-18 HPLC column (Supelco, Bellefonte, PA). Retinoids were eluted with 300 μ l acetonitrile and 42 μ l ddH₂O. One hundred μ l of sample was injected onto the column and allowed to separate for 20 min. Likewise, the treated cells were sonicated in 200 μ l of 0.25M ammonium acetate buffer pH 4.0, 4HPR and 330 μ l acetonitrile added, and processed by solid phase extraction, as described above. No degradation of retinoids was observed. Protein was determined by the Bradford method [44]. A calibration curve was generated for each experiment using purified atRA, ROH, 13-cis RA, and 9-cis RA (Figure 3C).

Retinaldehyde (RAL) and 13-cis-RA coelute in the above-described system. In order to distinguish between these two compounds, samples were divided in two equal aliquots and run using a slightly less acidic mobile phase. Adjusting the mobile phase to 80:2:12:0.5 acetonitrile:methanol:2 % ammonium acetate:glacial acetic acid resulted in an adequate separation of all-trans-retinaldehyde and 13-cis-RA on the same column as above.

Statistical analysis

Statistical analysis was done using Student's t-test.

Results

CRBP1 expression is lost in dysplastic lesions of human ovary

We examined the expression of CRBP1 protein by immunohistochemistry (IHC) in surgical prophylactic oophorectomy specimens obtained from asymptomatic women at high risk for breast and ovarian carcinoma and compared it to CRBP1 staining in control/background risk ovaries. The intention was to verify whether the loss of CRBP1 is a very early step in ovarian carcinogenesis, as suggested by our previous results [32]. Gross histology revealed that some of these sections contained various abnormalities alongside normal surface epithelia, such as deep surface invaginations (Figure 2A), inclusion cysts (Figure 2B), epithelial hyperplasia and papillomatosis. These epithelial morphologic changes in the ovarian specimens have been considered preneoplastic by some investigators [45–51]. As shown in Figure 2A and B morphologically altered epithelia exhibited lack of immunostaining for CRBP1, whereas normal surface epithelia of the ovary and ovarian stroma stained positive for CRBP1 (Figure 2C). This finding was consistent in the examined cohort. Ovarian stroma consistently stained positive for CRBP1 in all samples. No inclusion cysts and deep clefts were observed in the ovarian samples from control women at background/no known increased risk for ovarian cancer (Figure 2C).

Retinoic acid is produced in normal ovarian surface epithelial, but not in malignant cells

To test the hypothesis that there is a correlation between the loss of CRBP1 expression and the lack of RA synthesis in ovarian cancer, we conducted an HPLC analysis in cell lines in which CRBP1 expression was analyzed by real-time PCR (Table 1). Four independent cultures of HOSE cells were treated with retinol and both the cell supernatant and cell extracts were analyzed for retinoids. All-trans retinoic acid was detected in the culture medium of all four HOSE cell lines (Table 2). One HOSE cell line, HOSE1, exhibited highest levels of atRA production. Figure 3 shows representative chromatograms. All-trans retinoic acid was the major metabolite produced, however there was an unidentified peak eluting near 13-cis-RA's retention time (10.17 min) (Figure 3A-F). This was seen in all cell lines tested and probably does not represent true 13-cis-RA, but a nonenzymatic isomerization product [52]. Two of the HOSE cell lines also produced 9-cis-RA in their culture medium (Table 2). Interestingly, we were unable to detect any intracellular RA (at the detection limit of 8.2 pg/μl), as found in other studies, in which the metabolites are supposedly secreted into the culture medium [5,52]. As shown in Figure 3A, there was little intracellular retinol detected in treated HOSE cells. This low level appears to be due to efficient conversion of retinol into RA in normal cells. To determine that the RA in the culture medium was not the result of extracellular degradation of retinol, we removed and centrifuged medium from cultured HOSE cells. This cell-free medium was then treated with 2 μM retinol. No RA was detected, while 100% of retinol was recovered. This indicates secretion of RA from HOSE cells [5]. In addition, no atRA synthesis was seen in the medium of untreated HOSE, suggesting that retinoids were synthesized de novo. One of the two tested SV40-immortalized HOSE cell lines, HOSE118, was able to produce both atRA and 9-cis-RA, however no detectable retinoid production was seen in any of five ovarian carcinoma cells (Table 2). We were able to detect modest rises of intracellular retinol in retinol-treated ovarian cancer cell lines (Figure 3E). Exposure of A2780 to 2 μM retinol increased intracellular retinol levels from undetectable to 24.0 ± 8.6 pmol retinol/μg protein (n=3). Increasing retinol dose from 2 μM to 4 μM did not produce detectable RA in media or cell extracts, even though retinol uptake was higher (9% vs. 19%). Other carcinoma cell lines showed similar patterns of retinol uptake (data not shown). HOSE1 and HOSE118 were used for subsequent studies as representative of high and medium atRA-producing cells, respectively.

Oxidation of retinaldehyde is defective in human ovarian cancer cells

To confirm that the second oxidation step in RA production was defective in carcinoma cells, we measured the capacity of ovarian cells to produce the intermediate retinaldehyde (RAL) (Figure 4). It was observed using the mobile phase developed by Sakhi et al. that a RAL standard co-separated with 13-cis-RA [42]. Therefore, in order to separate the two retinoids, we decreased the concentration of acetic acid in the mobile phase, resulting in a more basic mobile phase. Using this system RAL separated from 13-cis-RA with elution times of 10.771 versus 11.754 minutes for RAL and 13-cis-RA. Table 3 reveals that all cell types were capable of oxidizing retinol to retinaldehyde, suggesting that the first reversible oxidation step was intact. There was a slight, yet statistically significant 35% increase in retinaldehyde production by normal HOSE1 over HOSE118 cells. Incubation of cells with vehicle ethanol produced no RAL in the medium. Inset in Figure 4 shows the chromatogram of A2780 culture media after retinol exposure. Normal, transformed and ovarian carcinoma cell lines were not able to produce 13-cis-RA.

Expression of retinoid-related genes is altered in human ovarian cancer cells

To understand the molecular defect contributing to the metabolic block, the expression of genes involved in RA signaling, transport, and production were analyzed in normal, transformed, and malignant ovarian cell lines (Figure 5). The rate-limiting step in the synthesis of RA is catalyzed by the enzyme RALDH. The major isoenzyme, RALDH2, is found in all RA-producing tissues, while RALDH1 has less specificity for this reaction. While all examined ovarian cell types expressed RALDH1, only cells capable of RA production, HOSE1 and HOSE118, expressed RALDH2. All malignant ovarian cell lines were negative for RALDH2 expression. To support this data, a preliminary IHC analysis for RALDH2 (ALDH1A2) was performed on normal (n=2) and Stage III serous ovarian adenocarcinoma (n=2) specimen. As shown in Figure 6A, surface epithelium stained positive for ALDH1A2. There was no staining in tumor cells (Figure 6B). These data suggest a major defect in the rate-limiting step of RA synthesis in ovarian carcinogenesis and also confirms findings in breast and prostate cancers [5,6,53]. Semi-quantitative PCR revealed expression of the retinoic acid nuclear receptor RAR α in normal, transformed and malignant cells, although its expression in OVCAR3, SKOV3, and PEO1 was significantly higher. There was abundant and constitutive expression of RXR α in normal HOSE, immortalized HOSE118 and all ovarian carcinoma cells, with the exception of A2780. In addition, CRABP2 was over-expressed in ovarian carcinoma cell lines relative to normal HOSE and HOSE118. There was no RAR β expression found in any of the cell types (data not shown), confirming findings by Zhang et al. [54]. Together these results show that although ovarian carcinoma cells express RA receptors and RA binding proteins, they have lost the capacity for RA production via loss of RALDH2 and decreased CRBP1 expression.

Sensitivity of ovarian cells to retinoic acid

In order to ascertain the growth effects of retinoids produced by HOSE cells, we determined the proliferative effects of atRA and retinol on normal, transformed and tumorigenic ovarian cell lines. Therefore, for comparison purposes, we used subphysiologic, physiologic, and pharmacologic concentrations of retinol and atRA, which are commonly used in the literature. In HOSE and HOSE118 cells, 3 μ M ROH treatment resulted in 20% growth inhibition (not significant at $p < 0.05$) (Figure 7a). However, this inhibition did not appear dose dependent. Growth in both normal and transformed cells was inhibited in a dose dependent fashion by atRA, as well as in the atRA-sensitive OVCAR3 and OVCAR10 cell lines (Figure 7b). These data suggest that although HOSE cells are responsive to exogenous atRA, the RA produced by pharmacologic doses of retinol was unable to further inhibit growth.

Discussion

We have previously identified that CRBP1, a protein crucial to proper vitamin A homeostasis in the cell, is consistently lost in the rat model of ovarian cancer, human ovarian cancer cell lines and in microdissected ovarian tumor tissues [31,32]. The finding of the loss of CRBP1 protein expression in potential precursor lesions in prophylactic oophorectomies in the current study strengthens our hypothesis that CRBP1, or a function related to this gene, is involved in the transformation process of the ovarian epithelium. As CRBP1 has been implicated in retinoid homeostasis, in RA production, and its expression regulated by RA, we hypothesized that ovarian tumor cells would have diminished retinoic acid production during the process of transformation.

Here we present the first evidence that HOSE cells are capable of synthesizing RA from retinol. More importantly, the surface epithelium loses this ability throughout the process of transformation, with ovarian carcinoma cells completely devoid of this capability. The metabolic block appeared to occur at the rate-limiting step, conversion of RAL to RA. This metabolic loss has been described in breast [5,53] and in prostate tumor progression [6,7]. In our studies, RA production was lost irrespective of CRBP1 expression, indicating that CRBP1 is not critical for this metabolic pathway. CRBP1 expression showed a similar pattern in breast to that of the ovary in our study.

The metabolic block leading to loss of RA-production in ovarian cancer cells appears, in part, to be due to a complete loss of the rate-limiting enzyme RALDH2 (ALDH1A2). Our results are similar to findings by Rexer et al, in which breast cancer cells, devoid of RA synthesis, were null for the RALDH isozyme ALDH6. RA production was restored when recombinant ALDH6 and CRBP1 proteins were added to MCF7 cell extracts in an in vitro reaction [41]. Interestingly, despite having lost the ability to produce RA, ovarian carcinoma cells still expressed cellular retinoic acid-binding proteins, as well as RA nuclear receptors (Figure 5). However, RAR α and RXR α were found to be highly expressed in archived human ovarian tumor samples [55], confirming our results where both retinoid receptors and CRABPs were abundantly expressed in the malignant phenotype.

It is unclear as to the impact that loss of RA production by OSE would have on early ovarian oncogenesis, given the pleiotropic actions of RA on growth, differentiation and apoptosis [56–58]. Indeed, atRA has been shown to induce growth arrest in the CA-OV3 and OVCAR3 ovarian carcinoma cell lines, although most other ovarian cancer cell lines were found to be resistant to RA-induced growth arrest [59]. Although atRA can inhibit normal and immortalized HOSE proliferation, it is not convincing that the RA produced from physiological levels of retinol in these cells would lead to growth arrest. Oxidative stress can be caused by buildup of toxic aldehyde. Therefore, the loss of the ability to generate RA from RAL may lead to oxidative stress in OSE.

In summary, our results suggest that ovarian oncogenesis is associated with altered CRBP1 expression and vitamin A homeostasis. Further in vivo studies, employing CRBP1-knockout mice under vitamin A restricted diet, may uncover if abnormal utilization of retinol in normal OSE cells leads to alterations in differentiation state, proliferative capacity, redox status and ultimately cancer.

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Figure 1.

Schematic of retinol metabolism to retinoic acid and associated binding proteins. Retinol is usually bound to CRBP1 inside the cell, protecting retinol from degradation. The first oxidation step is reversible however the conversion of retinal to retinoic acid is an irreversible reaction. Isomerization of all-trans retinoic acid, by a yet unidentified isomerase, is believed to generate the 9-cis retinoic acid isomer.

Figure 2.

The ovarian tissue specimens from women at high (A, B) and background risk (C) for breast and ovarian carcinoma were analyzed for CRBP1 expression by IHC. Epithelia lining deep surface invaginations (A) and inclusion cysts (B) exhibited no immunostaining for CRBP1. CRBP1 expression in normal ovaries (C): examples of sections of surface epithelium staining positive with anti-CRBP1. Note that brown color refers to CRBP1 staining. Slides were lightly counterstained with hematoxylin. Original magnifications: $\times 10$ (A, B) and $\times 40$ in the corresponding insets; (C) $\times 40$.

Figure 3.

HPLC analysis of retinol metabolism in normal, immortalized and malignant ovarian epithelium. Cells were treated with 2 μ M all-trans retinol for 16 h. Representative HPLC curves of cell extracts (A, C, E) and culture media (B, D, F) for HOSE1 cells (A, B), HOSE118 (C, D), and A2780 ovarian carcinoma cells (E, F) are shown. IS = internal standard. HPLC calibration curve for retinoids: sensitivity of HPLC detection was $\sim$ 300 pg/ μ l for all retinoids. This corresponded to an ability to detect 0.5 nM retinoids in the 2 ml of media. AUC=area under the curve.

Figure 4.

HPLC analysis of retinaldehyde: separation of 13-cis RA and retinaldehyde standard. 250 ng of both retinaldehyde and 13-cis RA were resolved by reverse-phase HPLC and detected using electrochemical detection. The more basic mobile phase allowed enhanced separation of retinaldehyde from other retinol metabolites. Elution times in minutes are shown above the corresponding peaks. Inset: HPLC of media from retinol-treated A2780 cells. A2780 cells were treated with 2 μ M all-trans retinol for 16 h. Media was analyzed for retinaldehyde according to Materials and Methods. Arrows denote retention time for retinaldehyde and 13-cis RA standards. IS=internal standard.

Figure 5.

Alterations of retinoid-related genes: retinoic acid receptor α (RAR α), retinoid X receptor α (RXR α), cellular retinoic acid-binding protein 1 and 2 (CRABP1 and CRABP2), and retinaldehyde dehydrogenase 1 and 2 (RALDH1 and RALDH2), in human ovarian cells. Semi-quantitative PCR was performed on normal (HOSE), SV-40 transformed (HOSE118), and various malignant (A2780, A1847, OVCAR3, OVCAR10, SKOV3, PEO1) cell lines. PCR for RAR β is not shown, as all cells were negative for RAR β expression. β -actin was used as loading control. Results are representative of two independent RNA isolates.

Figure 6.

ALDH1A2 (RALDH2) is expressed in the normal ovarian surface epithelium (A) but lost in malignant epithelium (B). Note that brown color refers to ALDH1A2 staining. Slides were lightly counterstained with hematoxylin. Original magnifications: $\times 10$, $\times 40$ in the corresponding insets.

Figure 7.

Sensitivity of normal, transformed, and neoplastic ovarian epithelial cells to retinol and retinoic acid. Normal human ovarian epithelial (HOSE), transformed HOSE (H118), and carcinoma cell lines (A2780, OVCAR3, OVCAR10, PEO4) were treated with various concentrations of all-trans retinol and atRA every four days over a 12 day period. Cell growth was measured by MTS assay (Promega) and represented as percentage of ethanol-treated controls. a) cells treated with all-trans retinol and b) cells treated with atRA. Results represent mean $\pm$ SE, n = 6, *p<0.05, **p<0.005.

Table 1

Real-time PCR analysis of CRBP1 expression in human ovarian cell lines

Cell lines	CRBP1 C _t (mean±SE)	GAPDH C _t (mean±SE)	Fold CRBP1 Decrease from HOSE1
HOSE1	27.42±2.62	21.20±0.68	
HOSE2	29.46±0.06	22.75±0.63	1.4
HOSE3	31.90±0.38	21.82±0.20	0.5
HOSE4	34.28±0.36	28.57±1.96	0.3
HOSE118	28.93±0.15	20.96±0.47	4
A2780	36.13±0.28	19.63±0.17	1137
SKOV3	32.17±0.56	20.12±0.10	59
OVCAR3	N.D. ^a	20.07±0.30	N.D.
OVCAR10	N.D.	21.22±0.85	N.D.

^aN.D. = not detectable

n=3

Table 2

HPLC analysis of vitamin A metabolism in human ovarian cell lines

Cell	Lines	all-trans RA (nM)	9-cis RA (nM)
Normal	HOSE1	203.4±135	28.1±9.8
	HOSE2	48.6±8.3	11.7±11.7
	HOSE3	28.5±1.7	ND
	HOSE4	21.1±2.5	ND
SV40-immortalized	HOSE118	42.8±16.3	29.6±17.3
	HOSE113	ND	ND
	A2780	ND	ND
Malignant	A1847	ND	ND
	SKOV3	ND	ND
	OVCAR3	ND	ND
	OVCAR10	ND	ND

ND = not detectable

 4×10^5 cells were treated with 2 μ M all-trans retinol for 16 h

Results (mean±SEM, n = 3) represent nM in culture medium

Table 3
HPLC analysis of retinaldehyde in human ovarian cell lines

Cell	Lines	Retinaldehyde (nM)
Normal	HOSE1	140.0±1.5 [*]
SV40-immortalized	HOSE118	103.0±1.5
Malignant	A2780	107.8±1.3
	OVCAR3	101.8±2.0

4×10^5 cells were treated with 2 μ M all-trans retinol for 16 h Results (mean±SEM, n = 3) represent nM in culture medium

^{*}
p<0.05