Award Number: DAMD17-01-1-0048

TITLE: Molecular Markers of Retinoid Action in Human Prostate

PRINCIPAL INVESTIGATOR: Rong Li, Ph.D.
Lorraine J. Gudas, Ph.D.

CONTRACTING ORGANIZATION: Weill Medical College of Cornell University
New York, New York 10021

REPORT DATE: June 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
4. TITLE AND SUBTITLE
Molecular Markers of Retinoid Action in Human Prostate

6. AUTHOR(S)
Rong Li, Ph.D.
Lorraine J. Gudas, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Weill Medical College of Cornell University
New York, New York 10021

E-Mail: rol2002@med.cornell.edu

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. ABSTRACT (Maximum 200 Words)
Retinoic acid (RA) has been used successfully in cancer prevention and therapy. RA exerts its biological effects through retinoic acid receptors (RARs, α, β, γ). It has been reported that RARβ plays an important role in mediating growth inhibitory actions of RA. The expression of RARβ is lost in prostate cancer cell lines, PC-3, and DU-145, while transfection of RARβ into PC-3 cells results in an increased sensitivity to growth inhibitory effects of RARβ againist. Despite the correlation between the level of RARβ and the RA-associated growth inhibition, it remains unknown how RARβ mediates the growth inhibitory effects of RA. This study used murine F9 wild type (Wt) and RARβ knockout (F9 RARβ−/−) cells as an experimental model to investigate the molecular mechanisms by which RARβ mediates the growth inhibitory actions of RA. Our study demonstrated that p27, a cell cycle progression regulatory protein, is increased by RA in F9 Wt cells as compared to the F9 RARβ−/− cells. In addition, RA stabilizes the protein stability of p27. Considering the striking findings that transfection of RARβ into the PC-3 cells results in an increased sensitivity to growth inhibition caused by RARβ against, our study may lead to more efficient chemotherapy with retinoids.

14. SUBJECT TERMS
Prostate, retinoic acid, retinoic acid receptor, p27

15. NUMBER OF PAGES
18

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT
Unclassified

18. SECURITY CLASSIFICATION OF THIS PAGE
Unclassified

19. SECURITY CLASSIFICATION OF ABSTRACT
Unclassified

20. LIMITATION OF ABSTRACT
Unlimited

NSN 7540-01-280-5500
Introduction

Prostate cancer is the most common cancer and the second leading cause of cancer deaths in males in the United States. Retinoids (retinol and its metabolites and derivatives) have been used in the prevention and treatment of some types of cancer. It has been shown that retinoic acid (RA), a biologically active form of retinol, is effective in inhibiting the cell growth and promoting differentiation of prostate cancer. It exerts its biological activities by binding to nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs). There are three RARs and three RXRs encoded by different genes (α, β, γ). Each RAR and RXR gene encodes several protein isoforms, generated by different promoter usage or alternate splicing. The RARβ2 isoform, the most abundant RARβ isoform, is transcriptionally induced by RA in many cell types (1). A limitation to designing effective retinoid therapies in the treatment of prostate cancer is the lack of understanding of the molecular mechanisms that control retinoid-mediated growth inhibition and differentiation. It has been reported that prostate cancer cell lines PC-3 and DU-145 do not express RARβ, while stable expression of RARβ into the PC-3 cells results in an increased response to growth inhibition mediated by a RARβ agonist and a hexafluoride vitamin D3 analog (2). There are data indicating that RARβ plays an important role in mediating the growth inhibitory actions of RA. Conversely, the loss of RARβ expression occurs during the process of carcinogenesis. Reduced expression of RARβ is a common feature of premalignant lesions and carcinogenesis. (3-21). Malignant cells with decreased expression of RARβ become resistant to RA treatment (15, 22, 23), whereas the up-regulation of RARβ parallels RA-induced growth suppression in some tumor cells (24-26). In this study we studied the mechanisms by which RARβ mediates the growth inhibitory actions of RA by using murine F9 wild type (F9 Wt) and F9 RARβ2 knockout (F9 RARβ2−/−) cells as an experimental model.

Body

We have previously shown that the F9 teratocarcinoma RARβ2 knockout cell line exhibits no growth arrest in response to RA, whereas F9 Wt, F9 RARα−/− and F9 RARγ−/− cell lines do growth arrest in response to RA. To examine the role of RARβ2 in growth inhibition, we analyzed the cell cycle regulatory proteins affected by RA in F9 Wt and F9 RARβ2−/− cells. Flow microfluorimetry analyses revealed that RA treatment of F9 Wt cells increased the percentage of cells in the G1/G0 phase of the cell cycle. In contrast, RA did not alter the cell cycle distribution profile of RARβ2−/− cells. In F9 Wt cells, cyclin D1, D3 and cyclin E protein levels decreased, while cyclin D2 and p27 levels increased after RA treatment. Compared to the F9 Wt cells, the F9 RARβ2−/− cells exhibited lower levels of cyclins D1, D2, D3, and E in the absence of RA, but did not exhibit further changes in the
levels of these cell cycle regulators after RA addition. Since RA significantly increased the level of p27 protein (~ 24-fold) in F9 Wt as compared to the F9 RARβ2−/− cells, we chose to study p27 in greater detail. The p27 protein plays a pivotal role in the regulation of the proliferation and differentiation of many cell types. Down-regulation of p27 has been observed in carcinogenesis and metastasis and the level of p27 has been used to evaluate cancer progression (27). The p27 mRNA level and the rate of p27 protein synthesis were increased in RA treated F9 Wt cells, but not in F9 RARβ2−/− cells. Moreover, RA increased the half-life of p27 protein in F9 Wt cells. Reduced expression of RARβ2 is associated with the process of carcinogenesis and RARβ2 can mediate the growth arrest induced by RA in a variety of cancer cells. Using both genetic and molecular approaches, we have identified some of the molecular mechanisms, such as the elevation of p27, through which RARβ2 mediates these growth inhibitory effects in F9 cells.
RA Results in Cell Growth Arrest in F9 Wt but not in F9 RARβ2-/- Cells

Figure 1A. Analysis of the growth of F9 Wt and RARβ2-/- cells after treatment with 1 μM RA. The cells were plated in duplicate wells at a density of 3000 cells/well. The cell numbers were counted on the indicated days. The experiment was performed three times with very similar results. The values represent the mean ± S.D. of three independent experiments.
Treatment with 1 μM RA for 96 hours. The values represent the mean ± S.D. of three independent experiments. * P > 0.05.

Figure 1B. Statistical analysis of the cell cycle distribution of F9 WT and RARβ²⁺ cells after RA treatment. RA increases the percentage of cells in G1 phase in F9 WT but not in F9 RARβ²⁺ cells.
RA Altered Cell Cycle Regulatory Proteins in F9 Wt and F9 RARβ2−/− Cells

<table>
<thead>
<tr>
<th></th>
<th>F9 Wt</th>
<th>F9 RARβ2−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Time (hrs)</td>
<td>24</td>
<td>24 48 72 96</td>
</tr>
<tr>
<td>cyclin D1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cyclin D2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cyclin D3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cyclin E</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDK 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDK 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDK 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>actin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2. F9 Wt and RARβ2−/− cells were treated with 1 μM RA for the times indicated. Total cell lysates were prepared and Western blot analysis was performed. The experiment was performed three times with each antibody with similar results. Actin was used as a loading control.
RA Increases the Synthesis of p27 Protein in F9 Wt but not in F9 RARβ2-/- Cells

<table>
<thead>
<tr>
<th></th>
<th>F9 Wt</th>
<th>F9 RARβ2-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Time</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>24</td>
</tr>
<tr>
<td>p27</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

![Graph showing [35S]-Methionine Radiolabeled p27](image)

Figure 3. (A) F9 Wt and RARβ2-/- cells were treated with 1 μM RA for the times indicated and then labeled with 50 μCi/ml [35S]-methionine for 30 minutes. Immunoprecipitation with anti-p27 antibody was performed. The radiolabeled protein precipitates were electrophoresed on a 10% SDS-polyacrylamide gel that was subjected to autoradiography. (B) The amount of signal in A was quantified by NIH Image. The experiment was performed three times with very similar results.
RA Increases the Level of p27 mRNA in F9 Wt but not in F9 RARβ2−/− Cells

A

<table>
<thead>
<tr>
<th></th>
<th>F9 Wt</th>
<th>RARβ2−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA</td>
<td>-  +  +</td>
<td>-  +  +</td>
</tr>
<tr>
<td>Time (hrs)</td>
<td>24 24 48</td>
<td>24 24 48</td>
</tr>
<tr>
<td>p27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>actin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

![Graph showing expression levels](image)

Figure 4. (A) F9 Wt and RARβ2−/− cells were treated with 1 µM RA for 96 hours. Total cellular RNA was extracted and Northern blot analysis was used to detect the level of p27 mRNA. (B) The amount of signal in A was quantified by NIH Image. The relative expression level of p27 mRNA was depicted as the ratio of the density of p27 mRNA to actin mRNA for the same time point. This experiment was performed three times with very similar results.
RA Stabilizes p27 Protein in F9 Wt Cells

Figure 5. F9 Wt cells were cultured in the absence or presence of 1 μM RA for 48 hours, pulse-labeled with 100 μCi/ml [35S]-methionine in the presence or absence of RA for 1 hour and chased for 6 hours. Immunoprecipitation with anti-p27 antibody was performed. The protein precipitates were electrophoresed on a 10% SDS-polyacrylamide gel that was subjected to autoradiography. The amount of p27 in the absence or presence of RA was analyzed with ImageQuant. The amount of p27 in the absence or presence of RA immediately after the 1 hour pulse labeling is set at 100. * P < 0.05.
Key Research Accomplishments
- Examined the effects of RA via RARβ on the protein levels of several cell cycle regulatory proteins, one of which is p27.
- Investigated the effects of RA via RARβ on the levels of p27 mRNA and protein.
- Determined the effects of RA via RARβ on the stability of p27 protein.

Reportable Outcomes
Delineated some of the molecular mechanisms by which RARβ mediates the growth inhibitory effects of RA.

Conclusions
The increase of p27 is associated with the growth inhibition induced by RA via RARβ in F9 WT cells. Considering the striking findings that stable transfection of RARβ to PC-3 cells results in a sensitivity to growth inhibition caused by RARβ against and a vitamin D3 analog, these data may be of use in designing more efficient chemotherapy with retinoids.

Reference


In addition to what I previously reported, I studied the effects of retinoids and lecithin:retinol acyltransferase (LRAT) on the differentiation of human prostate cancer cells. LRAT is an enzyme involved in the metabolism of retinol to retinyl esters. It has been reported that the levels of LRAT and retinyl esters are reduced in some human cancers, such as prostate. The human prostate cancer cell line PC-3 was transfected with LRAT. The functional activity of LRAT in all the transfected cell lines was determined by HPLC (Figure 1). All the transfected cell lines took up and esterified retinol into retinyl esters, while PC-3 wild type cells did not. The PC-3 and PC-3/LRAT transfectant cells were treated with retinoic acid (RA) or retinol (ROL) for various times. RT-PCR was used to test the effects of retinoids and LRAT on several molecular markers of retinoid action, such as keratin 18 and Gbx2, in human prostate. Soft agar assays for tumor cell growth were also performed.

Our data showed that there were no obvious changes in the levels of the above molecular markers upon RA or ROL treatment in both PC-3 and PC-3/LRAT transfectant cells (Figure 2). These findings are important both in basic and clinical research. They indicate that retinyl esters are not crucial ligands for the regulation of the above genes in the carcinogenesis of human prostate. Our studies provide new information about retinoid effects on prostate cancer cells and provide a rationale for more efficient chemotherapy with retinoids.
Retinol esterification in PC-3 Wt and PC-3/LRAT transfectants

Figure 1. Retinol esterification in PC-3 wild type and PC-3/LRAT transfectants.
<table>
<thead>
<tr>
<th></th>
<th>PC-3</th>
<th>PC-3/LRAT21</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>RA</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rol</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hours</td>
<td>24</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Keratin 18</td>
<td>48</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>Gbx2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 2.** The effects of LRAT on the differentiation markers of human prostate.