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13. ABSTRACT ***(Maximum 200 Words)***

This project is a study of the potential role of retinoic acid receptor in the metastasis of prostate cancer. Using prostate cancer cell lines, we found that the RXR-alpha expression was highly correlated with retinoid sensitivity. Clinical samples showed a trend towards a decreased expression in metastatic cells compared to the primary tumor cells. To determine whether the retinoid receptor RXR-alpha was directly involved in determining retinoid sensitivity, we prepared an adenovirus transducing RXR-alpha was created. Cancer cell lines were transduced with the adenovirus to overexpress the gene. These RXR-alpha transduced cells were showed no change in the sensitivity to retinoids in vitro. In addition we developed an experimental model of prostate cancer bone metastasis in nude mice. In this model, the transduction of the RXR-alpha by the adenovirus vector had no effect on bone metastasis. We conclude that in the experimental systems used, RXR-alpha is a marker of retinoid sensitivity but does not confer retinoid sensitivity or alter metastatic potential.

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Introduction

This project originated from extensive preclinical literature on retinoids, namely that retinoic acids, a group of derivatives of vitamin A, have growth inhibitory effect in many solid tumors, including prostate cancer, in various cell line models and animal models, as well as human trials. The best known examples are the use of retinoic acids in the treatment of acute promyelocytic leukemia, cutaneous T cell lymphoma and neuroblastoma [1-3]. In prostate cancer, many cell lines exposure to a retinoid induces in terminal differentiation, loss of cell proliferation and/or acquisition of a more mature phenotype of that particular lineage [4-8]. In most cases, these retinoid treated cell lines are no longer malignant. Finally, there have been clinical trials published on the use of retinoids or retinoid-enhancing compounds, generally in hormone-refractory, metastatic prostate cancer. For example, Trump et al treated a small number of patients with hormone refractory prostate cancer with all trans retinoic acid, and found no response, possibly because of altered pharmacokinetics during repeated dosing that led to low serum levels of the retinoid used [9]. Pienta et al used fenretinide, a retinoid whose target receptor is unknown, in patients with elevated PSA but negative biopsy, and observed no clinical effects after a short period of follow up [10].

In addition, we had made an observation that RXR-alpha, appeared to be a marker of retinoid sensitivity, namely that those cells which were RXR-alpha positive also happened to be retinoid sensitive. Since RXR-alpha is one of the retinoid receptor proteins, our observation raised an important possibility that RXR-alpha might not only be a marker of retinoid sensitivity but also a mediator of sensitivity. Furthermore, in solid tumors other than prostate cancer, RXR-alpha expression appeared to be decreased in metastatic tumor cells. We have compared expression of two retinoid receptors, RAR-alpha and RXR-alpha, in primary and metastatic tumor cells from cancer patients and have discovered that at least two of the retinoid receptors are decreased in metastatic tumor cells compared to tumor cells in the primary site. In addition, we have found that for one of these receptors, the loss of the receptor expression is due to a block at the level of mRNA translation. This raised a possibility that metastatic cells were no long resistant and/or that metastatic ability could be regulated by retinoids (i.e, retinoid sensitive cells have reduced metastatic ability).

Thus, the project sought to demonstrate whether RXR-alpha receptor was involved in retinoid sensitivity and/or metastatic ability.

Key Research Accomplishments

- Showed that RXR-alpha protein is expressed in retinoic acid sensitive cell lines but is not expressed in retinoic acid resistant cell lines.
- Found a wide range of RXR-alpha expression in clinical samples of prostate cancer regardless of the stage of cancer
- Found a difference in the level of RXR-alpha expression in breast cancer cells compared to normal epithelial cells
- Found that RXR-alpha transduced cells have the same sensitivity to retinoids as non-transduced cells in vitro
- Found that the transduction of the RXR-alpha by the adenovirus vector with or without retinoid treatment did not have any effect on bone metastasis

Conclusions

The major conclusions of this project are:

- RXR-alpha receptor expression appears to be a good marker of sensitivity prostate cancer cell lines to retinoids, but RXR-alpha receptor expression per se is insufficient for retinoid sensitivity, since retinoid-resistant cells engineered to express the RXR-alpha receptor remain resistant to retinoids

- RXR-alpha receptor expression does not appear to correlate with metastatic ability of prostate cancer cells, since the metastasis of prostate cancer cell line models in mice is unaffected by the transduction of the RXR-alpha gene.

These in vitro findings in mouse models and in patient specimen underscore the difficulties of extrapolating animal data to human disease. Based on these data, it appears that retinoic acids alone may not be effective in the prevention of prostate cancer metastasis. In the last 2-3 years phase I and II clinical trials have been completed on the use of retinoic acids in various clinical settings in prostate cancer, and retinoic acids has shown only modest benefit [11-14]. These clinical data appear to complement our cell line and mouse data.

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Supporting Data

1. Retinoic acid receptor expression

We studied the expression of RXR-alpha and RAR-alpha protein in prostate cancer cell lines and in patients with prostate cancer. We used immunohistochemistry using antibody directed against the RXR-alpha protein and the RAR-alpha protein. Standard formalin fixed, paraffin embedded sections were studied. For cell lines, western blotting was also used. The current studies extend the results reported previously and have determined that as a general rule, RXR-alpha protein is expressed in retinoic acid sensitive cell lines but not in retinoic acid resistant cell lines. This raised an important possibility that (a) RXR-alpha protein expression may be a marker of retinoid sensitivity, and that (b) RXR-alpha protein expression may directly confer sensitivity to retinoids.

To extend these findings, we studied the expression of the RXR-alpha protein in the cancer cells of patients with prostate cancer by immunohistochemistry. Unlike the results seen with cell lines, the patients do not show such a clear-cut separation of the expression level. Rather, there is a wide range of expression, primarily centered at "low-moderate" level of expression, regardless of the type of cancer, such as the Gleason grade.

As a comparison, an identical study was conducted in breast cancer patients. In breast cancer, there was a difference in the level of expression of RXR-alpha, as well as RAR-alpha, another retinoid receptor in the tumor cells compared to normal epithelium and in the metastatic tumor cells compared to the tumor cells in the primary site.

2. Construction of Adenovirus Vector Expressing RXR-alpha

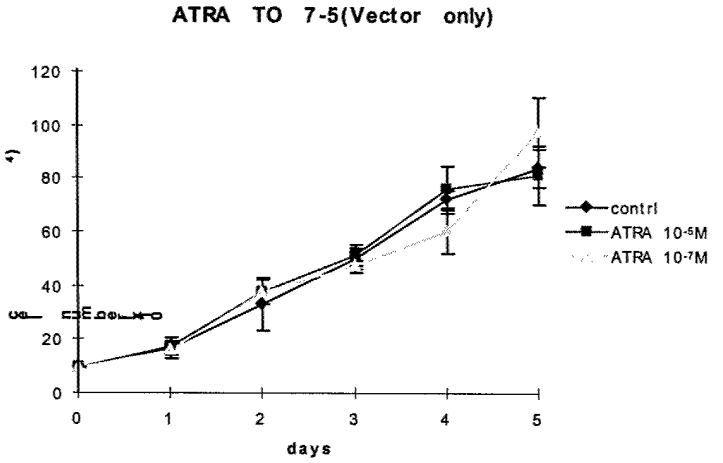
In order to address the question of whether RXR-alpha receptor is directly involved in retinoid sensitivity, we constructed a human adenovirus vector able to transduce the RXR-alpha gene. This vector allows us to determine the effect of RXR-alpha expression in a wide range of cell lines and in clinical samples in vitro. A modified replication-deficient adenovirus vector was inserted with the human cDNA for RXR-alpha in the E1A region. After screening for the appropriate recombinants, a single clone was isolated which proved to be the correct clone.

The vector was initially characterized using test cell lines. The vector was grown in 293 cells engineered with E1A, and purified to a high titer. This vector was then tested in cell lines, and gave the expression of the protein of the correct size. By adjusting the multiplicity of infection, the protein expression could be increased to a level corresponding to 100 fold greater than cell lines with endogenous expression of RXR-alpha.

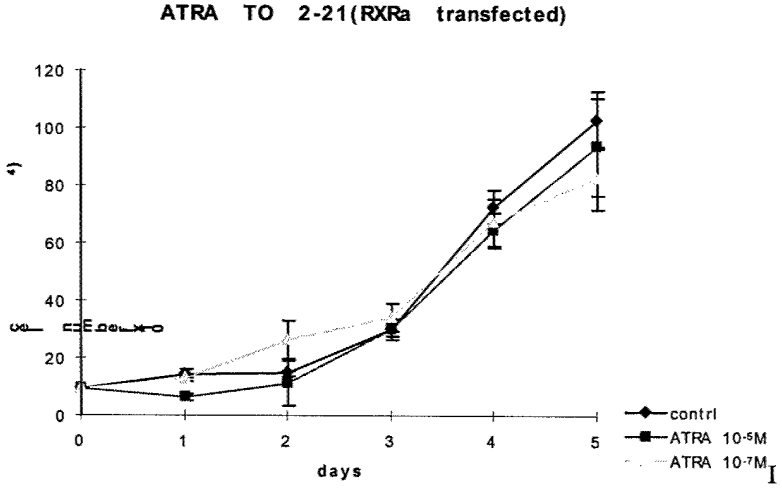
3. Effect of Transduction of RXR-alpha in Prostate Cancer Cell Lines

Transduction of the retinoid-resistant RXR-alpha negative cell lines with the RXR-alpha engineered vector resulted in the overexpression of the RXR-alpha. Varying the multiplicity of infection allowed adjustment of the levels of protein expressed, so that it was possible to select the level of RXR-alpha protein expressed. These cells remained viable with normal growth characteristics.

These cells were then treated with retinoids. The retinoids tested were: 13-cis retinoic acid, 9 cis retinoic acid, all trans retinoic acid, and LGD1069 (targretin). These were all used at the concentration of 1 μM. The cells remained viable and with normal growth characteristics in the presence of retinoids. As a comparison, similar studies were conducted in human breast cancer cell lines. After transduction of the cell lines with the RXR-alpha transducing adenovirus, there were also no differences in the sensitivity to retinoids.



Cell number versus time in the presence or absence of retinoic acid ATRA. The cell used is a retinoid-resistant cell line transduced with the control adenovirus vector.



Cell number versus time in the presence or absence of retinoic acid ATRA. The cell used is a retinoid-resistant cell line transduced with the RXR-alpha transducing adenovirus vector. This indicates that RXR-alpha transduction did not alter the sensitivity to retinoids.

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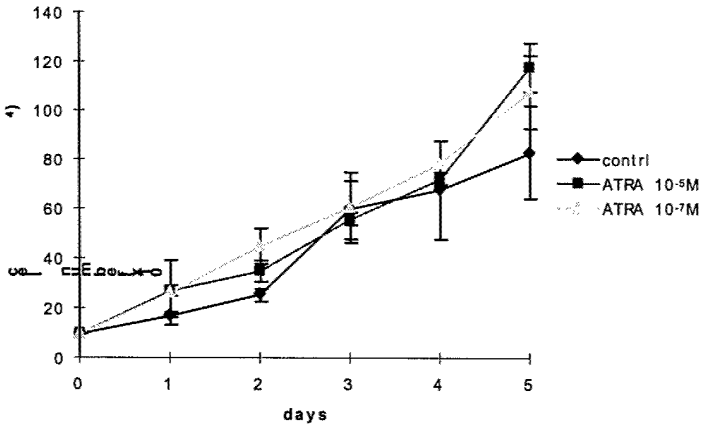
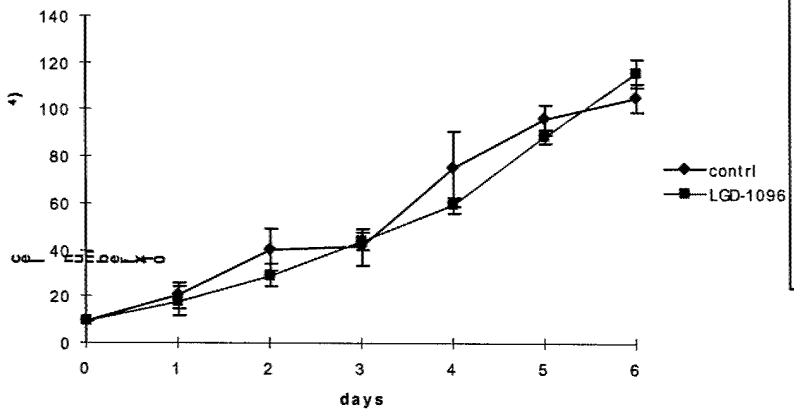


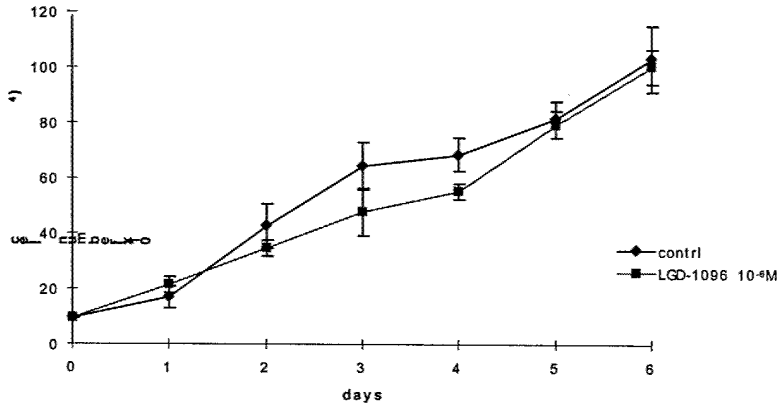
Cell number versus time in the presence or absence of retinoic acid ATRA. The cell used is a retinoid-resistant cell line.

LGD-1096 to 2-21

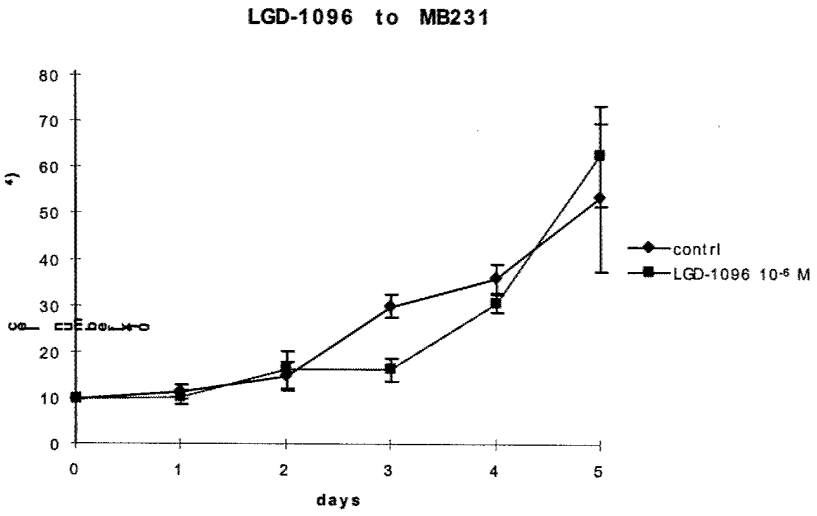


Cell number versus time in the presence or absence of retinoic acid LGD1069. The cell used is a retinoid-resistant cell line transduced with the RXR-alpha transducing adenovirus vector. This indicates that RXR-alpha transduction did not alter the sensitivity to LGD1069.

LGD-1096 TO 7-5(Vector only)



Cell number versus time in the presence or absence of retinoic acid LGD1069 (RXR specific). The cell used is a retinoid-resistant cell line transduced with the control adenovirus vector.



4. An assay to detect human cells in a bone metastasis model in mice

In preparation for experiments to determine the effect of retinoids on bone metastasis, we developed a method to quantify the number of human cells present in a background of mouse cells and to induce bone metastasis.

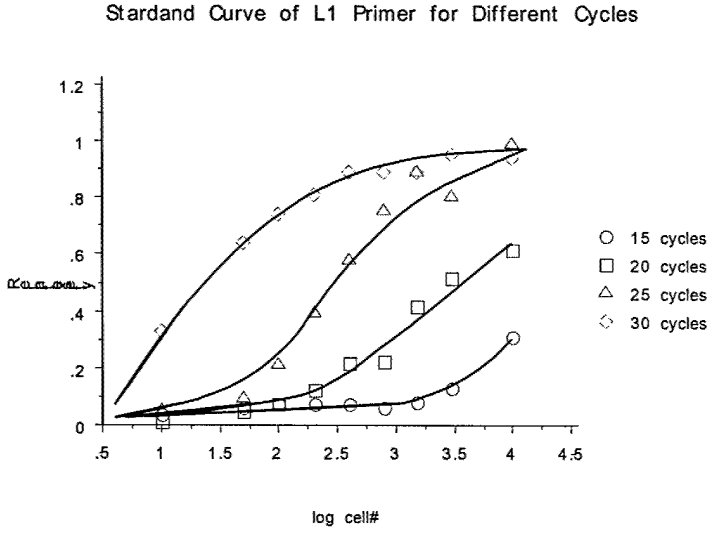
The quantification method involved assaying for a human repetitive sequence L1 in genomic DNA. This L1 element is present at about a few hundred thousand copies in the human genome but not present in the mouse genome. An example of such an experiment is shown below.

Figure 7 Detection of human repetitive sequence L1 in a mixture of human and mouse cellular DNA

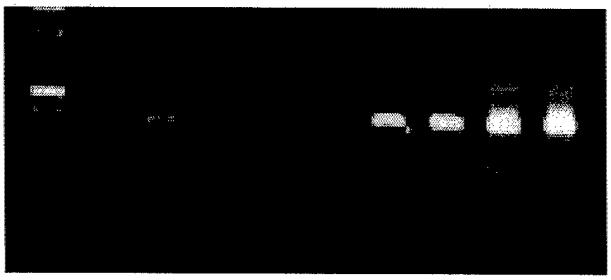
A known amount of human cell was spiked into 100,000 mouse cells, DNA extracted, and then PCR performed for L1 sequence using L1 specific primers.

From left to right,
no DNA; primers only;0 human cells, 10, 50, 100, 200, 400, 800,
1500, 3000, and 10,000 human cells in 500,000 mouse cells; 100,000 human cells

The intensity of the PCR product was proportional to the number of human cells present in the background of mouse cells. By optimizing the number of PCR cycles, a calibration curve could be prepared (see below).



The experimental system involved intracardiac injection of human tumor cell lines into the left ventricle of nude mice. After 2-3 weeks, the mice were sacrificed, and the bones crushed with mortar/pestle to isolate DNA located within bones. Quantitative PCR was performed on the DNA using primers specific for human repetitive sequence L1 (see above Figure). This allowed us to detect the minute amounts of human DNA present within mouse DNA with a high degree of sensitivity and specificity, in a quantitative manner.



Mice were injected with human prostate cancer cells and then assayed at 3 weeks. The femur was isolated, crushed with a mortar, and the DNA isolated. The human L1 PCR was performed to amplify human sequences.
This experiment demonstrates the feasibility of the technique. First lane (after the marker lane) is negative control (mouse DNA only). The last lane is human DNA only. All others are randomly selected mice injected with human prostate cancer cells.

5. Effect of retinoids in a bone metastasis model

In order to determine the effect of retinoid administration on bone metastasis, we developed a method of detecting bone metastasis in an experimental model. A known quantity of cell line (about 100,000) was injected into the left ventricle of the nude mouse. At 2-3 weeks later, the animals were sacrificed, and the bones from various locations were dissected and DNA isolated. The DNA was then assayed as described above to quantify the amount of human DNA present.

Prostate cancer cell lines known to be sensitive to retinoids were treated with various retinoids and then injected into the nude mice. Untreated cells served as controls.

At 3 weeks, no significant differences in the quantity of human DNA present in the mouse bones were detected between retinoic acid-treated and untreated cells.

6. Expression of A Gene Which May be Involved in Retinoid Resistance

In order to elucidate the mechanism of resistance to retinoids, despite the expression of appropriate receptors, we examined the expression of genes known to modify the retinoid sensitivity. Thus far, the data indicate that TGIF gene, which blocks the binding of RXR-alpha protein to its target and which interferes with SMAD activity, is expressed in the retinoid resistant prostate cancer cell lines.

7. Publications, manuscripts

A manuscript is now in preparation detailing the results from items 1-5 listed above.