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13. ABSTRACT (Maximum 200 Words)

The goal of this Prostate Cancer Center Initiation Award is to examine signal transduction pathways involved in prostate cancer progression, with an eye toward translational research applications. The program has two Projects and a Core Animal Facility. The first project (Dr. Carey) is focused on crosstalk between receptor tyrosine kinases and the androgen receptor (AR), using the Her2/neu kinase as a model system. Progress in the first year includes characterization of the IGFR pathway in xenograft models, preclinical studies with an EGFR/Her2-neu kinase inhibitor and biochemical analysis of AR-associated proteins by mass spectroscopy. During years 2 and 3, we anticipate further progress toward a better understanding of AR crosstalk and more insight into potential therapeutic strategies that can be applied clinically. The second project (Dr. Cohen) examines the role of IGF binding protein 3 (IGFBP-3) in the context of crosstalk with the retinoic acid co-receptor RXR α . Progress to date shows that IGFBP-3 induces apoptosis in synergistic fashion with RXR ligands (consistent with biochemical analysis showing that IGFBP-3 binds RXR). The leading hypothesis to be tested in years 2 and 3 is that IGFBP-3 acts as a partner of RXR in the nucleus leading to enhanced signaling towards apoptosis. The Core facility has provided mice for the preclinical therapeutic studies for both projects.

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Introduction

Overview

The goal of this Prostate Cancer Center Initiation Award is to examine signal transduction pathways involved in prostate cancer progression, with an eye toward translational research applications. The current funded program has two Projects and a Core Animal Facility. The first project, directed by Dr. Carey, is focused on crosstalk between receptor tyrosine kinases and the androgen receptor (AR), using the Her2/neu kinase as a model system. The second project, directed by Dr. Cohen, examines the role of IGF binding protein 3 (IGFBP-3) in the context of crosstalk with the retinoic acid co-receptor RXR α . The Core facility, directed by Dr. Sawyers, provides xenograft tissue and mouse models for preclinical therapeutic studies for both projects. Dr. Sawyers is also a collaborator for both projects. Please note that Dr. Cohen's project has been renumbered Project 2 (it was numbered Project 3 in the original application), as the original Project 2 was not recommended for funding.

Project 1 (Carey)

In the early phases of androgen-independence, genes traditionally regulated by androgen receptor are activated. How does this occur and what are the clinical ramifications? A cogent argument can be made that receptor tyrosine kinase (RTK) signaling pathways lead to modifications of AR or its co-activators, which in turn permit AR to function in a ligand-deprived environment. In cell culture, the androgen receptor can be activated independent of ligand by diverse stimuli including the tyrosine kinases Her-2/neu/Erb-B2, IL-6R, EGFR/Erb-B1, and IGF-1R (reviewed in Culig, 1998 and discussed in Craft et al., 1999). This project examines the hypothesis that there is a common theme in ligand-independent activation of AR using the receptor tyrosine kinases Her-2 and IGF-1R as models to pursue the mechanism. The study is a multidisciplinary collaboration with Dr. Charles Sawyers (Her-2) and Dr. Pinchas Cohen (IGF-I/IGF-1R).

Our proposal articulated a plan to systematically test predictions of the RTK signaling hypothesis. First, the signaling should be blocked by interfering directly with RTK function and this should in principle revert tumors to ligand-dependence. Second, because AR is the proposed endpoint of the pathway, then its biochemical alteration represents an important step in cancer progression. A direct effect of this pathway on association of co-activators with AR was proposed. Specific Aim #1 comprised two components: whether a RTK signal from IGF-I/IGF-1R employs the same strategy as we had previously deduced for Her-2 by Craft and colleagues (Craft et al, 1999) for enhancing progression to androgen-independence. The second component was to employ inhibitors of Her-2 (i.e., the herceptin antibody and EGFR kinase inhibitors) to determine if blockade therapy can inhibit signaling in cell culture systems and *in vivo*, i.e., revert tumors to ligand-dependence. Specific Aim #2, proposed to develop powerful biochemical and biological systems to analyze directly the effects of RTKs on AR isolated from tumors and genetically engineered cell lines based on the approach detailed in Huang and colleagues (Huang et al, 1999). Progress on both fronts was made although significant effort in Aim #2 was redirected to refine the protein analysis. The advantage of this change is to facilitate the scope and breadth of experiments to be performed in years 2 and 3 on cell line currently in development.

Project 2 (Cohen)

The goals of this project are to identify and characterize the role of IGFBP-3 in androgen receptor-signaling in androgen-dependent and -independent prostate cancer models. We are examining if IGFBP-3 is essential for apoptosis in response to androgen withdrawal. We are testing the effects of androgen deprivation on IGFBP-3 induction, IGF/IGF-R suppression, and prostatic apoptosis, in the AI and AD LAPC-4 and -9 cell lines and xenograft models of prostate cancer and in genetically altered mouse models. We are attempting to induce prostate cell death by treating these mice with systemic IGFBP-3. We are also trying to examine the role of RXR-alpha in IGFBP-3 actions in the prostate. We have begun to generate prostatic RXR-alpha conditional (*cre-lox*) knockout models in order to evaluate the prostates of these mice before and after castration or IGFBP-3 treatment. These experiments will determine if RXR is required for apoptosis in response to androgen deprivation. We are also studying IGFBP-3 synergism with ligands of RXR-alpha and related receptors both *in vivo* in the AD and AI LAPC models and *in vitro* in prostate cancer cell line models.

Body

Project 1 (Carey)

Changes in the IGF pathway during progression to androgen independence

Several lines of investigation have suggested that insulin-like growth factors (IGFs) are involved in the biology of prostate cancer, but little is known about their relevance to progression to androgen independence (AI). We used three *in vivo* models of androgen-dependent human prostate cancer to study this issue, in collaboration with Dr. Michael Pollack's lab in Canada. Progression to AI growth was associated with a 60-fold increase in expression of IGF-I mRNA in LAPC-9 xenografts and a 28-fold increase in IGF-I expression in LNCAP xenografts, relative to the initial AD neoplasms. *IGF-IR* mRNA levels were ~2.5-fold and ~5-fold higher respectively in AI LAPC-9 and LNCaP tumors compared to the original AD neoplasms. AI growth of these xenografts was also associated with significant reductions in IGFBP-3 expression. LAPC-4 xenografts, which previously have been shown to exhibit molecular pathology related to HER2-neu expression with progression to AI, showed relatively minor changes in expression of the genes investigated, but we nevertheless found evidence of increased IGF-IR phosphorylation with progression to AI in this model. Taken together with prior observations, our results suggest that deregulation of expression of genes related to any one of several critical receptor tyrosine kinase regulatory systems, including cellular IGF signaling, may confer androgen independence (Nickerson et al, submitted).

Evidence for selective requirement of the EGFR pathway in androgen-independent prostate cancer

We previously showed that signaling through the epidermal growth factor (EGF) receptor family can affect androgen receptor activity and growth in human prostate cancer (Craft et al, 1999). Here we asked whether interruption of ErbB-1 receptor signaling by an ErbB-1 receptor kinase specific inhibitor affects growth of human prostate cancer xenografts in our *scid* mice model (Klein et al, 1997). PKI-166 is an orally administered, ATP-site specific, reversible inhibitor of the ErbB-1 receptor kinase domain. At a daily dose of 100 mg/kg po, this drug completely inhibits in nude mice the growth of subcutaneous xenografts derived from the EGF-R overexpressing cell lines A431 and NCI-H596. Drug levels in these tumors after administration of 100 mg/kg PKI-166 correspond to drug levels which are sufficient *in vitro* to block EGF-mediated tyrosine phosphorylation, EGF-induced c-fos induction, and growth inhibition.

Experiments with PKI-166 in our human prostate cancer animal model system suggests antiproliferative activity against certain "androgen-independent" human prostate xenografts, i.e. tumors growing in castrated male mice. No significant growth inhibitory activity was noted on matched sublines of these human prostate cancer xenografts grown in intact male mice. Unfortunately a significant fraction of castrated animals treated with PKI-166 at 100 mg/kg daily by nasogastric gavage experienced unexplained toxicity, including death, two to three weeks after treatment begin, whereas none of the castrated male mice receiving vehicle died. We have also generated molecular data in tumor tissue showing inhibition of EGFR signaling in A431 and in prostate cancer xenografts.

Over the next year we plan the following studies to solidify this hypothesis:

1. Confirmation of the efficacy of PKI-166 in the prostate xenograft model

A much larger xenograft study in androgen-dependent and androgen-independent sublines of LAPC-4 and LAPC-9 are ongoing. The early results appear to confirm the initial finding that PKI-166 can block the growth of androgen-independent (AI) but not androgen-dependent (AD) tumors. In addition, we have initiated studies of the combination of androgen ablation + PKI-166 in AD xenografts. Our model would predict that this intervention will substantially delay the emergence of AI tumor growth.

2. Further evaluation of the toxicity of PKI-166 in castrate male mice

The good news is that toxicity appears to be much less of an issue in the larger confirmatory study. Nonetheless, we are carefully measuring a number of variables, including chemistry panels, blood counts, peak and trough plasma concentrations of PKI-166 and body weight of the mice. Full autopsies are done after 3 weeks of treatment, and histology of liver, kidney, etc will be examined. Finally, we have an additional treatment group in the castrate male mice that includes PKI-166 + androgen supplementation to determine if any androgen add-back can rescue any toxicity or efficacy of PKI-166.

3. Characterizing molecular determinants of response to PKI-166 in prostate cancer

Our hypothesis is that androgen independent prostate cancer growth is driven by the EGFR pathway when hormone therapy (Lupron, casodex, etc) fails. The current data with PKI-166 support that hypothesis. In my mind, a critical next step in clinical application is to identify those AI prostate cancers that will be sensitive to PKI-166. We propose to do this in two ways:

- a) We will expand the number of AD/AI xenograft pairs tested to determine if our observation can be generalized broadly or is specific to certain prostate tumors. So far, we have seen responses in AI sublines from 2 of 2 lines. We have 5 others that we can test.
- b) We will compare the status of signal transduction through the EGFR/Her2 pathway and AR pathway in the matched AI and AD sublines, which are sensitive and resistant to PKI-166 treatment, respectively. This will be accomplished through surveys of the levels of EGFR, Her2, autophosphorylation of the receptors as well as downstream substrates. We will also perform microarray studies.

In summary, our preclinical findings with PKI-166 support the hypothesis that EGFR/Her2 pathway signaling plays a functional role in androgen-independent prostate cancer growth. The studies proposed

here will solidify these findings, define molecular correlates of response and form the basis for intelligent clinical trial design.

Identification of androgen receptor alterations that occur during progression to androgen independence

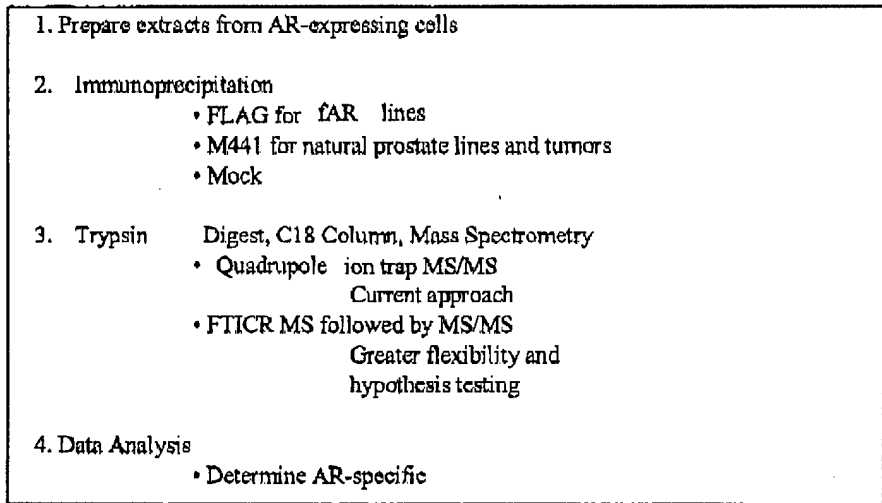
The focus of this aim was to probe the molecular alterations of AR when androgen signaling is replaced by RTKs. The ability to complete this aim is predicated on the development of techniques for efficiently analyzing AR, its modifications and its associated factors from cell lines and tumors. Our hypothesis is that signal transduction pathways regulate association of AR and its co-factors during the AD-AI transition. The goal of our proposal is to develop a methodology for identifying the AR-associated proteins participating in this transition. To develop this methodology, we have initiated a collaboration between with Drs. Michael Weber and Don Hunt at University of Virginia. The results of the collaboration and our data analysis website are described below. The project in years 2 and 3 will employ the technology to identify AR associated proteins in LNCaP cells expressing RTKs (Craft et al., 1999) and in natural xenograft tumors (Klein et al., 1997). Specific aspects of our work during the past year are described below:

1. The identification of AR modifications and associated proteins in AD and AI cancer through targeted proteomics.

A flow diagram of the approach is shown below. Our model for testing the technology is a HeLa cell line expressing fAR from an amphotropic retrovirus (Huang et al., 1999). We employed HeLa cells because they can be grown in spinner culture in 32-liter scale and yield 500 pmol of fAR. Although we began at this scale for practical reasons, the sensitivity of the procedure is in the 50-attomole range. FLAG-tagged AR is precipitated along with any associated proteins using FLAG antibody affinity resin. The advantage of this approach is that the fAR and associated proteins can be eluted from the resin with FLAG-peptide. However, an alternative approach involves immunoprecipitation with an AR monoclonal antibody, M441, and elution with glycine, pH 3.5. This variation has already been successfully applied to LNCaP cell lines and LAPC4 and LAPC9 tumors containing endogenous AR. Our controls for specificity include mock immunoprecipitations from extracts lacking FLAG-AR (in the case of FLAG antibody) or pre-immune Ig (in the case of M441). The entire protein mixture in the immunoprecipitate is digested with trypsin, and the resulting peptides are analyzed directly using liquid chromatography and mass spectrometry (LC/MS). Peptides are first separated in time by reverse-phase liquid chromatography followed by either data-dependent mass spectrometry (MS/MS) on a quadrupole ion trap mass spectrometer or high resolution mass analysis on a Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometer. Sequence information is then derived from MS/MS spectra by either database searching (SEQUEST program and NCBI non-redundant protein database). One advantage of MS is that it also permits identification of post-translational modifications (i.e., phosphorylation, acetylation). The last step involves comparison of peptides detected in the mock and fAR samples to identify unique AR binding proteins. In the first approach, data dependent MS/MS experiments and database searching yield a list of peptides (and parent proteins) contained in the mock and fAR samples. Proteins detected in the mock sample were then subtracted from proteins detected in the fAR sample to provide a list of candidate AR-associated proteins. We have developed a bioinformatics protocol for analyzing the samples, in collaboration with Drs. Ioannis Xenarios and David Eisenberg at UCLA. We have now obtained 3096 peptide sequences from the control and experimental samples. A website has been set-up to allow subtractive analysis of peptides and protein identified from mock and experimental samples. We will build more complexity to this site as the data from more relevant LNCaP and tumor samples begin to flow in. The website currently allowed us

to analyze the large amount of data from the MS experiments with HeLa fAR and identify relevant interacting proteins. The page can be found at http://dip.doe-mbi.ucla.edu/carey/Compare_Exp1_Exp2.cgi. The website returns a list of GiCode and the number of times the observation has been made. In summary the key AR-associated proteins that emerged from this analysis in HeLa are as follows: AR, Glucocorticoid receptor, Mineralcorticoid receptor, several heat shock proteins, several DEAD box proteins, a nuclear matrix associated protein and others (see appendix item #1 for a more complete list). We found few co-activator candidates of the type that were reported to interact with steroid receptors. The AR we employ is transcriptionally active in HeLa when reporters are co-transfected although we imagine that there are few genes influenced by AR in the HeLa cell. We are currently applying the technology to LNCaP cells and LAPC9 xenograft tumors where AR is known to be active. It is anticipated that more relevant hits will be identified.

Flow Diagram for Identification of AR-associated proteins



2. Analysis of AR/SRC-1 complexes in xenograft tumors and in stable Her-2 and IGF-I cell lines

To better optimize the androgen-independent effect of Her2 we systematically investigated the effects of different Her2 variants on PSA enhancer transcriptional activity. We found that the dominant active (DA) rat Her2, when transiently transfected into serum derived LNCaP cells, was able to stimulate the activity of PSA ARE containing promoter up to 30 fold, similar to the effect of 1nM R1881. We concluded that the DA-Her2 is

a better agent for analysis of the RTK effects. We have now transfected DA-Her2 into LNCaP cells and are in the process of growing up and analyzing the stable transfectants.

3. Reconstitution of AR/SRC-1 physical and functional interactions

Preliminary data indicated that Her2-overexpressing LNCaP cells permitted an AR-SRC-1 interaction in the absence of ligand. Our attempts to recreate this in reconstituted systems failed. Although the ligand dependent association of AR-SRC-1 could be recreated in 293T transfections, the Her2 effect could not. We employed the wild type human Her2 in this experiment but will employ the DA Her2 in a repeat.

Project 2 (Cohen)

IGF axis expression in relation to androgen status in LAPC-4 and -9 xenografts in SCID mice

Using a variety of approaches we have shown that androgens regulate IGFbps in prostate cancer xenografts. Looking at gene expression changes in LAPC-4 tumors grown in mice using the Affimetrix gene array system, we observed that IGFBP-3 was dramatically induced by castration in this model. These results were confirmed using immunoblotting studies on a series of tumors. Furthermore, upon reversion to an androgen independent state the expression of IGFBP-3 was suppressed similarly to the androgen replete state.

Creation of prostatic RXR knockout mice

We have obtained the LoxP-RXR and the Cre-Probasin mice and have initiated matings. We will begin to examine homozygous mice histologically before and after castration and will treat mice with IGFBP-3 and rexinoids and study prostates looking for apoptotic effects. These experiments are scheduled for the coming year.

Demonstration of IGFBP-3 interactions with additional regulators of prostate cancer

This year we have also demonstrated that IGFBP-3 is essential to the effects of vitamin D on prostate cancer cells and showed that IGFBP-3 affects P21/waf1 expression directly. In the coming year we will examine what role RXR and RXR ligands have on this effect. In addition we recently published that IGFBP-3 binds transferrin and showed that IGFBP-3 and transferrin have opposing effects on prostate cancer cell apoptosis. This novel finding explains, in part, the well-recognized survival effects of transferrin on cells that are unrelated to its iron carrying capacity. In recent experiments characterizing the molecular interactions of IGFBP-3 and RXR we observed that IGFBP-3 causes displacement of the orphan nuclear receptor TR3 (also known as NUR77/NGFI-B), which then translocates into the mitochondria directly inducing apoptosis. This constitute a novel mechanism of action for IGFBP-3 and explain some of its rapid effects on apoptosis.

IGFBP-3 and RXR synergism *in vitro*

We also showed (Fig. 2) that IGFBP-3 and the specific rexinoid LG1069 (from Ligand Inc.) had additive effects in killing LAPC-4 cells. This is an important observation as it suggests that IGFBP-3 and RXR ligands co-operate on inducing gene transcription that leads to apoptosis. Furthermore, these data suggest that these agents will have a synergistic effect on CaP tumors *in vivo* and thus may be useful as co-adjuvant therapy in prostate cancer.

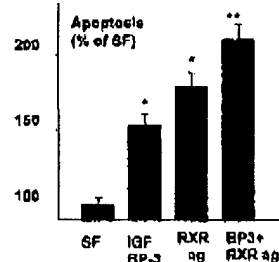


Fig 2. Synergistic effects of IGFBP-3 and RXR agonists on the killing of LAPC-4 prostate cancer cells. This experiments illustrates the potential utility of IGFBP-3 as a adjuvant therapy for prostate cancer

IGFBP-3 and RXR synergism *in vivo*

On going experiments are evaluating the effect of infusing IGFBP-3 alone or in combination with RXR ligands on the growth of LAPC-4 and LAPC-9 xenografts in SCID mice. Preliminary data shows that enhancement of local IGFBP-3 levels in such xenografts through the inactivation of IGFBP-3 proteases results in suppression of tumor growth (data presented in the 2001 AACR meetings).

Core Facility (Sawyers)

Under Dr. Sawyers' direction, the core facility has provided human prostate cancer xenograft material for study of signaling pathways in Projects 1 and 2. In addition the core facility has set up the tumor models for preclinical studies and assisted investigators in the conduct of these studies by measuring tumor volume and serum PSA levels. The RXR α knockout mice for Project 2 have just arrived, and the core facility will assist in genotype analysis. When ready, the core facility will also assist in crossing the mice to probasin-Cre strains and/or in direct injection of Cre into the prostate gland (Dr. Reiter and Dr. Lily Wu). Finally, the core handles tissue fixation, H&E staining and immunohistochemical analysis of xenograft and mouse tissues (Dr. Said, Pathology).

Key Research Accomplishments

Project 1

1. We shown specific activity of the EGFR/Her-2 tyrosine kinase inhibitor PKI-166 against androgen independent prostate cancer xenografts.
2. We have shown that IGF-I and IGF-IR expression and activity are altered in some xenografts during the transition to androgen independence.
3. We have developed cell lines expressing dominant active (DA) Her2.
4. We have developed a protocol for the efficient immunoprecipitation of AR from cell lines and tumors.
5. We have developed mass spectrometric paradigm for analyzing AR and its associated proteins.
6. We have validated the mass spectrometry by showing that AR is present and interacts predictably with proteins previously identified as AR interactors.
7. We have developed a mass spectrometry database to track the interactions

Project 2

8. We have identified a clear effect of IGFBP-3 on the induction of prostate cell apoptosis which is mediated by interactions with RXR
9. We have identified a synergistic effect of IGFBP-3 and RXR ligands on the killing of prostate cancer cells.
10. We have provided evidence for the involvement of IGFBP-3 in mediating the actions of Vitamin D, a nuclear receptor ligand that requires RXR homo-dimerization.
11. We have isolated a novel IGFBP-3 partner in the form of transferrin and demonstrated functional effects on prostate cancer cells.

Reportable Outcomes

Publications

1. Rajah R, Khare A, Lee PD, Cohen P. Insulin-like growth factor-binding protein-3 is partially responsible for high-serum-induced apoptosis in PC-3 prostate cancer cells. *J Endocrinol* 1999; 163:487-94.
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3. Grimberg A, Cohen P. Role of insulin-like growth factors and their binding proteins in growth control and carcinogenesis. *J Cell Physiol* 2000; 183:1-9.
4. Boyle BJ, Zhao XY, Cohen P, Feldman D. Insulin-like growth factor binding protein-3 mediates 1alpha,25-dihydroxyvitamin d3 growth inhibition in the LNCaP prostate cancer cell line through p21/waf1. *J Urol* 2001; 165:1319-24.
5. Weinzimer SA, Gibson TB, Collett-Solberg PF, Khare A, Liu B, Cohen P. Transferrin Is an Insulin-Like Growth Factor-Binding Protein-3 Binding Protein. *J Clin Endocrinol Metab* 2001; 86:1806-13.
6. Nickerson T, Chang F, Lorimer D, Smeekens SP, Sawyers CL, Pollack M. In vivo progression of LAPC and LNCaP prostate cancer models to androgen independence is associated with increased expression of IGF-I and IGF-IR, submitted.

Major Presentations:

- Nov 1999 The Fifth International IGF Symposium. Brighton, England.
Lecture: "IGFBP-3 in CaP: new partners, new functions".
- May 2000 HypoCCS Symposium, Sorrento, Italy.
Lecture: "IGF and Prostate Neoplasia"
- May 2000 The GRS consensus meeting on Growth Hormone Safety, Keswick VA.
Lecture: "GH, IGF and Prostate Cancer:"
- May 2000 The 6th International Growth Forum (IGF-VI), Boston MA.
Lecture: "The GH-IGF-Cancer connection"
- Sep 2000 The IGF Cancer Symposium, Halle Germany.
Lecture: "Roles of IGFBPs in prostate cancer pathophysiology"
- Sep 2000 Cap CURE Annual Meeting. Lake Tahoe, CA
Lecture: "IGFBP-3: a potential therapy for prostate cancer"
- Oct 2000 IGFBP2000 Symposium, Sydney Australia.
Lecture: "Nuclear IGFBPs in prostate cancer biology:"

Database: http://dip.doe-mbi.ucla.edu/carey/Compare_Exp1_Exp2.cgi

Translational research:

Based on our work on the EGFR axis in the LAPC model, we will begin a phase I-II clinical trial at UCLA and Dana Farber Cancer Institute testing the inhibitor of EGFR/Her-2 (PKI166, from Novartis) + leuprolide in men with hormone refractory prostate cancer.

We are currently planning studies that will involve treatment of men who have prostate cancer with IGFBP-3 peptide in combination with RXR ligands. These studies are scheduled to begin in 2004.

Cell lines: We have developed one novel prostate cell line, DAHer2-LNCaP which we are analyzing for activity.

Conclusions

This Prostate Cancer Center Initiation Award continues to examine signal transduction pathways involved in prostate cancer progression, with an eye toward translational research applications. Project 1 has shown the potential role of the EGFR/Her-2 pathway in androgen independence, with a clear path toward testing kinase inhibitors of this pathway in patients. The project also characterizes the role of RTK signaling prostate cancer progression to androgen independence in relevant cell culture and animal models using powerful proteomic analyses. Over the next two years we will gain a better mechanistic understanding of the role of RTKs in AI cancer from both a clinical and biochemical perspective. Project 2 has used innovative approaches to characterize prostate cancer pathophysiology as it relates to nuclear receptors and their interactions with IGFBP-3. The data show that IGFBP-3 is an important regulator of apoptosis in prostate cells, particularly in conjunction with RXR agonists. Since pharmacological modulation of this pathway is clinically feasible, we anticipate that this project will also lead to clinical trials.

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