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13. ABSTRACT (Maximum 200 Words) During phase I of this IDEA Award, we examined mechanisms of androgen independent prostate cancer progression using our LAPC xenograft model ^{1,2} . Our focus was to identify genes and/or signaling pathways that might be responsible for androgen-independent growth, through expression cloning. We have successfully identified several such candidates through screening xenografts and validated the activity of these candidates in xenograft models. Our current focus is to study two genes/pathways that were identified in this screen (the EGFR/Her2 pathway and the cathepsin D protease) as tools to create new transgenic models of prostate cancer. We are also developing a new transgenic model using the avian retrovirus receptor TVA that will allow us to introduce multiple transgenes into the prostate in a stepwise manner and to manipulate androgen levels without affecting transgene expression (as part of the Phase II grant).				
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Introduction

The goal of this research is to identify and characterize genes which are responsible for the progression of prostate cancer to androgen independence. Our strategy is to use a series of human prostate cancer xenografts established by us (LAPC 4 and LAPC 9) to isolate these genes through expression cloning. During the course of the past 2+ years, we completed task 1 (isolation of genes) and task 2 (characterization of genes in xenograft models). We are now working extensively on task 3 (characterizing genes in transgenic models), which will continue to be the focus of my Phase II award. After extensive screening we have chosen to focus on two genes/pathways - Her2-neu/EGFR and cathepsin D. The logic behind these choices has been addressed in previous progress reports. In each case, the biological characterization includes overexpression of the gene in androgen-dependent prostate cells and examination of the growth properties of those cells in vitro and in SCID mice. We are also examining the effects on signaling through the androgen receptor, as we believe this may be a major mechanism by which these genes cause androgen-independent growth. These points are best illustrated in the preprint describing a role for Her2/neu in prostate cancer³. Our focus for the remainder of the grant is to examine the effects of two of these gene pathways (Her2-neu/EGFR and cathepsin D) in the context of the mouse prostate gland through the creation of transgenic mice.

Body

Ligand-independent activation of the androgen receptor by EGFR/Her2 pathway signaling

Our work has implicated the EGFR/Her2 pathway and the intracellular kinase MEKK1 in ligand-independent activation of the androgen receptor. The details of these studies can be found in appendix material submitted with a prior progress report^{3,4} and will not be repeated here because of space limitations. The major conclusion from this work is that activation of either of these signaling pathways leads to ligand-independent AR activation, and both can be invoked as potential mechanisms to explain androgen-independent prostate cancer growth. Although very little is known currently about the expression of MEKK1 in clinical prostate cancer, there is a growing body of literature suggesting that activation of the EGFR signaling pathway (through overexpression of either Her2 or activated alleles of EGFR) occurs frequently in prostate cancer⁵⁻⁸. In the case of Her2, it is important to note that the mechanism is quite distinct from breast cancer. Most studies report that Her2 gene amplification does not occur in prostate cancer⁹ (and D. Slamon, UCLA, personal communication), although there are some exceptions¹⁰. Rather, it appears that Her2 protein is overexpressed in the absence of gene amplification in a fraction of early stage prostate cancers. The protein level tends to be lower than those found in Her2 amplified, Herceptin-responsive (also called 3+ Her2-positive) breast cancers. Recently, three groups have performed studies of metastatic and androgen-independent prostate cancers and find that up to one third of cases express elevated levels of Her2 protein (M. Loda, Dana Farber, personal communication; R. Cote, USC, personal communication); C. Cordon Cardo, MSKCC, personal communication).

Aberrant expression of EGFR and EGFR ligands: In addition to Her2, it is becoming clear that other components in the EGFR/Her2 pathway can be deregulated in prostate cancer. For

example, TGF α ligand and EGFR are co-expressed in androgen-independent prostate cancers⁸. Most compelling, however, is a recent report showing expression of the variant EGFRvIII receptor in a large fraction of advanced prostate cancers⁵. EGFRvIII is a ligand-independent mutant lacking much of the extracellular domain (due to deletion of exons 2-7) originally described in human gliomas^{11,12}. Taken together, our studies in the LAPC xenograft model and the analysis of EGFR and Her2 expression in clinical material suggest that dysregulation of this signaling pathway plays a critical role in some prostate cancers. We will test this hypothesis through the creation of transgenic mice expressing either Her2 or mutant EGFR in the prostate (as part of the Phase II Award).

Isolation of the serine protease cathepsin D in a screen for androgen independence genes: The observations implicating Her2 in androgen-independent prostate cancer came from educated guesses in a survey of tyrosine kinase signaling pathways that might be upregulated and could potentially affect AR signaling. In parallel, we devised a strategy to identify genes from androgen-independent prostate cancers which allow cells to grow in the absence of androgen by functional expression cloning. This strategy is unbiased since it screens an entire cDNA library rather than restricting the analysis to specific gene classes. We constructed an amphotropic retrovirus cDNA library from the androgen-independent stage of our LAPC-4 xenograft to allow high efficiency gene transfer and stable expression of cDNAs in target cells. We then used in vitro and in vivo screens to identify genes which could cause androgen-dependent LNCaP, LAPC-4 or LAPC-9 cells to grow as colonies in soft agar or as tumors in mice in the absence of androgen. Retroviral inserts were recovered from individual colonies or tumors by PCR and sequenced. Cathepsin D, a lysosomal aspartyl protease previously implicated in breast cancer progression, was isolated multiple times independently

Cathepsin D and the IGF pathway: Cathepsin D was originally identified as an estrogen responsive gene and is known to function as an oncogene in vitro and in vivo¹³⁻¹⁵. Cathepsin D is also implicated in apoptosis regulation in cells exposed to interferon¹⁶. A number of substrates for cathepsin D have been identified, but their precise physiological role in cathepsin-mediated bioactivity remains to be defined. Among the most compelling for prostate physiology is IGF binding protein 3 (IGFBP-3), which is cleaved by cathepsin D to liberate IGF-1. Multiple studies have established that cathepsin D and IGFBP-3 are both expressed in prostate epithelial cells. Interestingly, the PSA protease can function together with cathepsin D to cleave IGFBP-3 more efficiently¹⁷⁻¹⁹. In addition, IGFBP-3 is upregulated in prostate cancer cells after castration and is sufficient to induce apoptosis^{20,21}. Therefore, it is quite plausible that cathepsin D could positively affect prostate cancer growth by blocking the pro-apoptotic effects of IGFBP-3 and enhance signaling through the IGF pathway.

Overexpression of cathepsin D in androgen-dependent xenografts enhances progression to androgen independence. Having isolated cathepsin D multiple times in the expression cloning screen, we next asked if cathepsin D is sufficient to confer a gain-of-function phenotype in our prostate cancer xenograft models. First, we sequenced the cathepsin D alleles isolated in the screen and found no mutations, indicating that elevated levels of wild-type cathepsin D are responsible for the phenotype. Next we prepared high titer retrovirus expressing cathepsin D,

infected androgen-dependent LNCaP and LAPC4 cells and injected these cells into intact or castrated male SCID mice. The results show that cells expressing high levels of cathepsin D have a significant growth advantage in castrate animals but not in intact males. This finding is remarkably similar to our findings with Her2 overexpression³ and provides further confidence in the potential role of cathepsin D in androgen independence.

Transgenic mice expressing cathepsin D in the prostate develop prostatic hyperplasia and prostate cancer. To build upon our finding in the xenograft models and test the potential role of cathepsin D in prostate cancer initiation, we created transgenic mice expressing cathepsin D under the control of the probasin promoter. Six founder lines were derived, three of which show expression of the transgene in the prostatic lobes as measured by immunoblot. Immunoblot analysis of 4 of these lines (2 positive and 2 negative) using anti-cathepsin D antibodies. Remarkably, histologic characterization shows prostatic hyperplasia in all three transgene-expressing lines between 9-12 months of age and well-differentiated prostate carcinoma in one founder line at one year of age. Our future goals are to characterize these transgenic mice and define the mechanism by which cathepsin D confers androgen independent growth.

Key Research Accomplishments

1. We have identified the Her2/neu tyrosine kinase as a gene which can cause androgen independence.
2. We have identified the MEKK1 serine/threonine kinase as a gene which can activate the androgen receptor.
3. We have provided evidence for the origin of androgen independence through clonal evolution using xenografts developed by our group.
4. We have isolated cathepsin D from an expression cloning experiment and shown that overexpression in xenograft models causes androgen independence.
5. We have created transgenic mice expressing the cathepsin D serine protease specifically in the prostate gland as a model of androgen independence.

Reportable Outcomes

Publications:

1. Craft N., Shostak Y., Carey M., **Sawyers C.L.** (1999) A mechanism for hormone independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. Nature Medicine, 5(3):280-85.
2. Abreu-Martin M.T., Chari A., Palladino A.A., Craft N.A., **Sawyers C.L.** (1999) Mitogen activated protein kinase kinase kinase (MEKK1) activates androgen receptor-dependent transcription and apoptosis in prostate cancer. Mol. Cell. Biol., 19(7):5143-54.
3. Craft N. and **Sawyers C.L.** (1999) Mechanistic concepts in androgen-dependence of prostate cancer. Cancer and Metastasis Reviews, 17:421-27.
4. Craft N., Chhor C., Tran C., Belldgrun A., DeKernion J., Witte O.N., Said J., Reiter R.E., **Sawyers C.L.** (1999) Evidence for clonal outgrowth of androgen-independent prostate

cancer cells from androgen-dependent tumors through a two-step process. Cancer Research, 59:5030-36.

5. Huang W., Shostak Y., Tarr P., Sawyers C., Carey M. (1999) Huang W., Shostak Y., Tarr P., **Sawyers C.**, Carey M. (1999) Cooperative assembly of androgen receptor into a nucleoprotein complex that regulates the prostate-specific antigen enhancer. J. Biol. Chem., 274:25756-68.
6. Craft N., Wu X., Tran C., Raitano A., Sawyers C.L. (2001) Functional expression cloning implicates the cathepsin D protease in prostate cancer progression. Under review.

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 a small molecule inhibitor of EGFR/Her-2 (called PKI166, from Novartis) + leuprolide in men with hormone refractory prostate cancer.

Cell lines: We have developed two novel prostate cell lines, LAPC-4 and LNCaP/Her-2, and made these available to interested researchers.

Conclusions

This project has used innovative technologies to characterize prostate cancer progression to androgen independence in relevant models. We have chosen to focus on two genes/pathways implicated in this transition - Her2-neu/EGFR and cathepsin D – each of which is now being examined in transgenic models. The most immediate implication is that inhibitors of these signaling pathways may have therapeutic utility in men with prostate cancer when combined with traditional hormone therapy. These concepts are being explored in our preclinical models and in phase I-II clinical trials.

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