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13. ABSTRACT (Maximum 200 Words)

This Prostate Cancer Center Initiation grant has been designed to identify genetic and molecular markers of prostate cancer progression within and between racial ethnic groups (African-Americans, Latinos, Whites, Japanese) at substantially distinct underlying risk of prostate cancer. Our Epidemiology Core has obtained signed tissue releases from prostate cancer patients to date identified during follow-up of the Hawaii/Los Angeles Multiethnic Cohort study and has procured 438 tissue samples to date (163 African-Americans, 163 Latinos, 74 Caucasians, and 38 Japanese). These have been processed histopathologically by Project C, which has completed immunohistochemical staining for COX-2, p27, and Caveolin-1 markers with analysis of additional markers ongoing. Project B, studying the androgen receptor (AR) gene in detail, has conducted functional studies to understand non-steroidal activation of AR signaling. Project A, studying the SRD5A2 gene in detail, has characterized a series of the somatic mutations identified in tumors to date using site directed mutagenesis assays.

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Overall Introduction

We report below our third year progress in our Prostate Cancer Center Initiation grant. The overall goal of our Center is to understand molecular and genetic factors associated with progression of occult prostate cancer to invasive disease, as only a small subset of patients progress, but for which no mechanism currently exist to identify which ones. Moreover, the rate of progression appears to differ substantially among racial-ethnic groups as the prevalence of occult cancer is similar among African-Americans, Latinos, Whites and Japanese-Americans but the incidence of invasive disease varies several-fold across these same groups.

The Center consists of three Projects and one Core and we organize our progress report along these lines. The Core is designed to identify prostate cancer patients from a large prospective study in Los Angeles and Hawaii, obtain signed tissue release forms from these patients, secure tissue samples from hospitals and distribute these to the three Project laboratories. We had substantial delay in starting this critical aspect of the project because of IRB issues locally and at the Army Medical Research Center; these have all been resolved and all aspects of this part of our program have proceeded extremely well in the past year. We have had substantial success (see below) in identifying and characterizing sequence variants in the androgen receptor gene and the steroid 5-alpha reductase type II genes, the major goals of Projects B and A, respectively. Although the budget for Project C was reduced by more than 50% and much of the activity of this Project to date has been to process and characterize the tissue samples as they are sent by hospitals, we have nonetheless finished a substantial number of immunohistochemical studies of molecular markers of progression as planned in this Project including p27, COX-2 and Caveolin-1 with others ongoing. As all of the projects are ongoing and there is no renewal mechanism for this grant, we have been granted a one-year no cost extension to complete each of the projects as originally planned.

PROJECT A

THE HUMAN 5RD5A2 GENE AND PROSTATE CANCER PROGRESSION

Project A: The Human 5RD5A2 Gene and Prostate Cancer Progression
Principal Investigator: Juergen Reichardt, Ph.D.

INTRODUCTION

There is a large variation in prostate cancer rates between racial-ethnic groups in the US. We have taken a "candidate gene" approach to prostate cancer. We have focused on androgen-metabolic genes since they can regulate prostatic growth. Specifically, we proposed to examine the hypothesis that *de novo* DNA sequence variations (i.e. somatic mutations) in the type II (or prostatic) steroid 5α-reductase (SRD5A2) gene contribute substantially to the progression of prostate cancer particularly across racial/ethnic lines.

BODY

In our application we had proposed to investigate the following three interrelated specific aims:

1. To identify somatic mutations in prostatic tumors of men from four racial/ethnic groups [African-Americans, Asian-Americans, Caucasians and Latinos] in the regulatory elements of the SRD5A2 gene, specifically its promoter and the 5' and 3' untranslated regions (UTR);

2. To determine the frequency of somatic SRD5A2 mutations in prostate cancers in four racial-ethnic groups [African-Americans, Asian-Americans, Caucasians and Latinos];

3. To determine the contribution of the SRD5A2 somatic mutations screened for in specific aim 2 to prostate cancer grade and stage of disease as surrogates for outcome.

KEY RESEARCH ACCOMPLISHMENTS

Substantial progress was made toward specific aim 1 last year as reported. Furthermore, we had reconstructed all *de novo* somatic amino acid substitutions in the SRD5A2 cDNA last year. Therefore, we have begun to express them in *cos* cells to determine their biochemical properties. Specifically, we have assayed 6 mutants at the biochemical and pharmacogenetic level (Table 1). We note that there is significant biochemical variation in somatic mutations at this locus since the A49T and F118L mutations increase activity significantly (which we measured as the apparent V_{max}): 5-fold for the A49T, 4-fold in the case of the F118L and more than two-fold for the V3I mutation (Table 1). We also have uncovered great pharmaocogenetic diversity: the inhibition constant (apparent K_i) varies 36-fold for finasteride (from 5-180 nM (Table 1). We have also uncovered similar phamacogenetic variation for the other two drugs we examined: PNU and GG745 (Table 1). We made significant progress toward specific aim 2 as reported last year: we have already genotyped 87 DNA samples for the A49T (alanine at codon 49 replaced by threonine) and V63M (valine-63 to methionine) *de novo* somatic mutations. In addition, we identified two novel substitutions. We are currently pursuing them.

Table 1
Characterization Somatic Mutations in the SRD5A2 Gene

Mutation	V_{max} (nmol/min/mg)	K_M (Testosterone; μM)	K_M (NADPH; μM)	pH	K_i (finasteride; μM)	K_i (PNU; μM)	K_i (GG745; μM)
wt	2	0.6	8	6.0	60	6	17
V3I	5	3	12	5.5-6.0	27	3	93
A49T	9.8	1.5	7	6.0	180	1.1	1.1
V63M	1.6	1.5	11	6.0	38	10	36
F118L	8.2	4.5	17	6.0-6.5	5	1.7	4
V189A	0.8	1.3	39	6.0-6.5	90	12	23
G191E	0.9	1	25	6.0	40	18	25

REPORTABLE OUTCOMES

None thus far besides Table 1.

CONCLUSIONS

This laboratory has completed the sequencing component of specific aim 1. We are, therefore, poised to begin with the investigation of the biochemical properties of the somatic mutations we identified in the SRD5A2 locus. In specific aim 2, we have already begun screening for the A49T and V63M recurrent protein-coding mutations in tumor and "normal" samples obtained through this grant. Both were found repeatedly as outlined in Table 1. Genotyping will, therefore, continue in the next year and novel mutations will be identified by DNA sequencing (cf. Table 1). This strategy will allow us to determine their contributions to tumor progression in specific aim 3 in the coming year.

REFERENCES

None

APPENDICES

None

PROJECT B

ANDROGEN RECEPTOR (AR) SIGNALING

IN PROSTATE CANCER PROGRESSION

Project B: Androgen Receptor (AR) Signaling in Prostate Cancer Progression
Principal Investigator: Gerhard A. Coetzee, Ph.D.

INTRODUCTION

Androgen receptor (AR) activity is implicated in all phases of prostate cancer [1-3] including the final stages of disease that ensue following failure of androgen ablation therapy (AAT) which frequently is termed androgen-independent. Recent evidence suggests that prostate cancer cells surviving after AAT are not necessarily resistant to subsequent alternative hormone manipulations that depend on a functional AR. Continued signaling of the AR in a castrate hormone environment could result from overexpression of the receptor, gain-of-function AR gene somatic mutations, AR coactivator overexpression and ligand-independent activation of the AR. It was recently proposed that stimulation of the Janus kinase-signal transducers and activators of transcription (JAK-STAT) and/or mitogen-activated protein kinase (MAPK) pathways by interleukin-6 (IL-6) and forskolin activated the AR in the absence of ligand in LNCaP prostate cancer cells [4, 5]. Mechanisms for this possibly include phosphorylation of the steroid receptor coactivator-1 [6] and/or recruitment of the coactivator p300 [7]. While these mechanisms theoretically may account for the continued activity of the androgen-signaling axis following androgen ablation, their contribution to AR signaling and prostate cell growth *in vivo* is not known.

The expression of PSA is dependent on androgen signaling in prostate epithelial cells and has been used extensively as a marker of prostate cancer growth. The binding of transcription factors to two upstream cis regions, a proximal promoter and a distal enhancer, of the PSA gene results in transcriptional regulation. Two AREs are located in the promoter and six are located in the enhancer region some 4.2kb upstream from the transcription start site [8-10]. Recently chromatin immunoprecipitation (ChIP) experiments have revealed that AR complexes with both the PSA promoter and enhancer to mediate PSA expression via a ligand-dependent mechanism [11]. Since most of the work assessing androgen-independent activation of the AR so far has relied on transiently-transfected reporter genes, we developed a model system for androgen-dependent and -independent activation of the AR by targeting the endogenous chromatin-integrated PSA gene and measuring its mRNA expression by RT-PCR and promoter/enhancer occupancy by ChIP analyses in LNCaP cells. Using this model system we examined AR-mediated PSA expression and promoter/enhancer occupancy after AR activation with the natural ligand DHT, the cytokine IL-6, and the adenylate cyclase activator forskolin and unexpectedly found that IL-6 opposed the effects of DHT and forskolin on PSA expression.

BODY

Task 1: Our initial plan was to recruit and analyze a total of 480 prostate cancer tumor blocks. To date we have accrued only 119 and will attempt to obtain the balance in the period of no-cost extension during the next year.

Task 2: To enable a better analysis of AR structure and function, we have developed a novel chromatin immunoprecipitation (ChIP) assay to allow an analysis of AR function on natural gene promoters and enhancers. Our results were published recently (see below) and what follows is a short summary of our main findings.

Ligand-activated androgen receptors (ARs) occupy target genes and recruit histone modifiers that influence transcriptional competency. In LNCaP prostate cancer cells, the natural ligand 5α-dihydrotestosterone (DHT) activates transiently-transfected AR-responsive promoter constructs; concurrent treatment with the protein kinase A activator forskolin enhanced AR stimulation induced by DHT. Additional treatment with the cytokine IL-6, purportedly an AR activator, markedly inhibited receptor activity. To assess AR activity on natural chromatin-integrated promoters/enhancers, we determined AR occupancy of the endogenous prostate specific antigen (PSA) promoter/enhancer as well as PSA expression in LNCaP cells treated with DHT; AR occupancy of the PSA enhancer was rapid (within 1 hour of stimulation), robust (10-fold over background), and sustained (8-16 hours). In contrast, AR occupancy of the PSA promoter was only increased by 2-fold. Histone H3 acetylation at both the enhancer and promoter was evident 1-2 hours after DHT treatment. Detectable pre- and mature PSA mRNA levels appeared after 1 and 6 hours treatment, respectively. Substantial qualitative and quantitative differences in PSA expression and AR occupancy of the PSA enhancer were observed when DHT-induced and ligand-independent activation of the AR were compared; forskolin stimulated PSA mRNA and protein expression, whereas IL-6 inhibited both DHT- and forskolin stimulated expression. IL-6 did not diminish DHT-dependent AR occupancy of the PSA enhancer but inhibited CBP/p300 recruitment, histone H3 acetylation and cell proliferation. These findings provide a contextual framework for interpreting the contribution of non-steroidal activation of the AR to signaling *in vivo*, and have implications for prostate cancer cell growth.

KEY RESEARCH ACCOMPLISHMENTS

Whereas IL-6 and forskolin potentially can activate the MAPK signalling pathway, it appears that their effects on PSA expression in LNCaP cells occur via different mechanisms. In our system, IL-6 and forskolin had opposite effects on endogenous PSA expression. It is possible that the inhibitory effects of IL-6 observed in our study are mediated via non-MAPK mechanisms such as the JAK-STAT pathway. The MAPK and JAK-STAT pathways might therefore affect AR and/or coactivator and/or corepressor phosphorylation in different ways to elicit the opposite effects on AR-mediated gene expression. The challenge remains to disentangle such mechanisms in order to understand maintenance of AR activity in the prostate in a castrate or androgen ablated state. The endogenous PSA promoter/enhancer system described here provides a model system for such an elucidation

REPORTABLE OUTCOMES

Buchanan, G., Irvine, R.A., Coetzee, G.A., Tilley W.D.: Contribution of the androgen receptor to prostate cancer predisposition and progression. Cancer and Metastasis Reviews 20, 207-223 (2001).

Jia, L., Kim, J., Shen H., Clark, P.E., Tilley, W.D., Coetzee, G.A.: Androgen receptor activity at the prostate specific antigen locus: Steroidal and non-steroidal mechanisms. Mol. Can. Res. 1, 385-392 (2003).

CONCLUSIONS

The single main finding from work (supported by the present grant) is that the AR likely plays a vital role in prostate cancer progression even during androgen-independent phases of the disease.

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APPENDICES

None

PROJECT C

CELLULAR AND MOLECULAR MARKERS

OF PROSTATE CANCER PROGRESSION

Project C: Cellular and Molecular Markers of Prostate Cancer Progression
Principal Investigator: Richard Cote, M.D.

INTRODUCTION

Prostate cancer is a highly heterogeneous disease with an unpredictable course. Although the epidemiology and etiology of prostate cancer is largely unknown, it is a disease with extraordinary racial-ethnic variation in incidence, mortality, and survival. African-American men have by far the highest rates of prostate cancer in the world, whereas Asian men native to China, Japan and Korea have the lowest. Even for prostate cancers presenting at a specific stage, African-Americans have substantially worse survival, whereas Asian-Americans appear to have substantially better survival than whites including Hispanics. Indeed, a recent report shows that even in an equal-access medical care setting, prostate cancer survival for black men is poorer compared to white men, suggesting that the disease is particularly aggressive in black men.

The steps that a tumor must undergo to be invasive and metastatic (i.e. the critical factors leading to patient death) are becoming increasingly well characterized. These include:

- Loss of hormonal regulation that can also have important implications in the control of metastatic disease.
- Loss of cell cycle control: Loss of tumor suppressor function (e.g. p53, Rb, PTEN) that can have multiple effects on regulation of cell growth, angiogenesis, and the ability of a tumor to enter the cell death (apoptotic) pathway. Similarly, inactivation of cdk-inhibitors (p27, p21, p16) is expected to result in increased proliferation rates of tumor cells (as detected by PCNA, Ki67 and Topoisomerase II (expression).
- Loss of growth control: A number of groups have identified loss of function of the PTEN phosphatase as a common event, particularly in advanced prostate cancer. The primary consequence of loss of PTEN function is deregulation of the PI3-kinaseAkt pathway, which is oncogenic in many tumor models.
- The ability to form a new blood supply (angiogenesis), which is important in delivering nutrients and removing waste from a tumor, and also in providing a route for tumor metastasis. Loss of normal inhibitors of angiogenesis (thrombospondin-1) can lead to increased neovascularization (detected by microvessel density).
- Loss of normal cell matrix adhesion properties and cell-cell interactions (including contact inhibition), which allow tumor cells to grow past normal cell density and to break away from their primary site and form occult metastases, or overt metastases.

BODY

We are constantly searching for new markers that will help us address the biovariability of tumors among members of different racial-ethnic groups and novel technologies to improve analysis capabilites. To this end last year we identified two new markers to add to our test battery; Cox-2 and Caveolin-1. COX-2 is an isoform of cyclooxygenase and is an enzyme that metabolizes arachidonate to prostaglandin G2 and then to prostaglandin H2. Caveolins are major structural proteins of caveolae-specialized plasma membrane invaginations that are abundant in smooth muscle cells, adipocytes, and endothelium, and mediate signal transduction activities and

molecular transport (Harder et al, 1997). We discussed these markers in detail in our last Progress Report. Since we have numerous markers to analyze, we have developed, in collaboration with George McNamara, a multi-marker technique by which we can look at up to 4 or more different markers of biologic status on a single tissue section using spectral imaging techniques. Using this technique, we are now able to assess three to four different tumor markers on a single tissue section, thus multiplying our resources significantly. Until this technique was developed and optimized for use in our laboratory we were unable to test all of the markers proposed on the limited number of slides available to us.

This year our laboratory has acquired yet another novel and important asset for the interpretation of IHC markers. The Automated Cellular Imaging System (ACIS, ChromaVision, San Juan Capistrano, CA) has the potential of substantially improving the accuracy, sensitivity and reproducibility of immunohistochemical analysis. The ACIS system is based on the detection of colors and combines automated microscopy and computerized image processing to assist in precisely quantitating staining intensities and percent positively stained cells. We have acquired color threshold software that allows us a great deal of flexibility in utilizing this technology. We are currently developing ACIS parameters for the markers used in this study (see Key Research Accomplishments). The additional information supplied by this new technology will shed some important light on the future application of automated imaging in assessing tumor markers by IHC.

These new markers and novel techniques to maximize available tissue and standardize interpretation will better enable us to determine the relationship between the changes in these key biological pathways and a) race/ethnicity, b) age, and c) intermediate markers of tumor progression (tumor stage and grade). We will eventually be in a position to relate these changes to clinical outcome (survival and mortality across racial-ethnic groups).

It is our hypothesis that the difference in tumor behavior observed in prostate cancer arising in men of different racial groups has a molecular and cellular basis. We are obtaining patient specimens from a highly multiethnic population of Los Angeles, California in planning and executing this project. These studies are expected to provide information leading to a better understanding of prostate cancer progression in men of different racial/ethnic groups. While our study emphasizes racial/ethnic variability, it also addresses important issues concerning prostate cancer outcome for all men. Facts that predispose one group of men to have more aggressive tumor, may be predictive of behavior of prostate cancer in all men. Our initial focus is on known pathways of tumor progression, studying factors that have been shown to be important (or potentially important) predictors of prostate cancer behavior. We are blinded to the clinical stage and grade of the tumor as well as the patient's race.

KEY RESEARCH ACCOMPLISHMENTS

One of the major functions of this laboratory is to analyze the tissue from the submitting institutions for a variety of parameters. Initially the tissue is assessed for type, (i.e., resection, core biopsy or TURP). Each tissue section is then examined for the amount of tumor present and a Gleason score is given. To date we have received formalin-fixed, paraffin-embedded tissue from 429 cases of prostate cancer and entered these into the laboratory database providing them

with a laboratory number. This number is linked in our database to the patient's study identification number. We have assessed all 429 of these for the presence of tumor, for the percentage of tumor to normal prostate tissue and recorded the Gleason grade of the tumor in the slides provided. In most cases, sufficient tissue is available for immunohistochemical analysis. In addition, we have examined the tissue and assessed its suitability for DNA extraction. On specimens that have sufficient tumor for this purpose the areas of cancer are traced out on the H&E section, then copied on the back of an unstained section and colored in red. It is primarily resection samples with greater than 5% tumor that are suitable, core biopsy specimens generally do not contain enough tumor tissue for this type of analysis. This is done on two unstained sections per eligible case. The slides that have the area that contains tumor colored in red (non-tumor tissue is outlined in blue) are sent to Drs. Coetzee and Reichardt (Project A and B) for their analysis. This technique allows their laboratory to differentially remove tumor verses non-tumor on the slide. We have identified 184 of the 429 specimens that contain sufficient tumor for successful extraction. Tissue from 133 of these cases has been supplied to Dr. Coetzee (Project B) and Dr. Reichardt (Project A) for analysis, the remaining 51 will be sent to their laboratory within the next few weeks.

Our other major function is the immunohistochemical staining and analysis of specified markers. To date we have stained, reviewed and recorded the results of p27 on 252 cases, on 231 cases with antibody against COX-2 and on 75 cases using Caveolin-1. Nine additional cases were immunostained and found to be unsatisfactory for analysis due to technical issues. The additional markers that we still plan to assess include bcl-2, E-cadherin p53, Rb, CD34, p21, p16, Ki67, PCNA, Topoisomerase-II and thrombospondin-1. These markers have been optimized by our laboratory and are ready for application. The limiting factor is available tissue per case. The multiple marker analysis by Spectral Imaging will allow us to do most, if not all, of these markers listed on virtually every tissue with sufficient tumor present.

Subjectivity and inter-observer variation may play a role in the interpretation of certain markers. As discussed above, we now have in our laboratory the Automated Cellular Imaging System to detect, classify, and count cells of interest based on the recognition of cellular objects of particular color, size and shape using standard laboratory cell staining procedures. We have modified the detection parameters to suit each individual antibody used in this study. This system can determine the percentage and intensity of immuno-positivity in specific areas. These areas are specifically chosen by the pathologist and any number of fields or size of fields that are appropriate can be instantly analyzed once the slides are scanned. We are running standardization tests and will soon begin the side-by-side analyses of the tumor markers by both manual and automated methods.

REPORTABLE OUTCOMES

None

CONCLUSIONS

This study is a molecular epidemiologic study designed to elucidate multi-ethnic differences in prostate cancer progression. It takes an innovative approach to develop and apply

novel biologic markers of prostate cancer progression, and we are continuing to add novel techniques that will increase the information we can obtain from this study. We are applying novel technologies to this study that were not even available to us when the project was proposed. We are continuing to receive more case samples and are continuing to make significant progress in our goals to assess multiple, significant markers of disease progression we anticipate completing this study during our one year no cost extension.

REFERENCES

None

APPENDICES

None

EPIDEMIOLOGY CORE

Core: Epidemiology Core
Director: Brian E. Henderson

INTRODUCTION

This is a study looking at the differences in tumor behavior (molecular and cellular behavior) observed in prostate cancer arising in men of different racial groups. The specific aims of the project are to: (1) identify and contact incident prostate cancer patients diagnosed among participants in the Hawaii-Los Angeles multiethnic cohort study to obtain signed tissue release forms, (2) secure formalin-fixed tissues on these individuals; to process these samples; and to distribute these samples to laboratories involved, (3) develop and implement data forms to record laboratory results and histologic reviews; to conduct data management activities including data entry and editing, (4) Project A will try to determine the frequency of somatic mutations in the SRD5A2 gene, (5) Project B will try to determine the frequency of somatic androgen receptor gene mutations in prostate cancer and the functional significance of these, and (6) Project C will use immunohistochemistry to look at a panel of molecular markers thought to possibly be indices of progression.

Our data may partly explain the differing rates of progression from occult to clinically meaningful disease across racial-ethnic groups. These data may be useful in identifying prostate cancer cases who would benefit from improved treatment modalities based in part on somatic alterations in the SRD5A2 or AR genes in their tumors or the presence of other molecular markers of progression, and in identifying those occult lesions requiring the most (or least) aggressive therapy.

BODY

We continue to identify the newly diagnosed African-American and Latino-American prostate cancer cases in the multi-ethnic cohort. 1412 men have been identified and contacted by mail, and in some cases, by phone and asked to sign the tissue release forms. These consents were approved by the University of Southern California IRB office and sent to the men identified as having prostate cancer through follow-up linkages with our SEER cancer registry. 698 men (348 African-American and 350 Latinos) have signed the forms and returned them to us by mail. We are in the process of calling the other respondents to encourage them to sign and return the consent forms. We are also tracking cases through our cancer registry follow-up department, whose letter has been returned to us as undeliverable. Seventy-seven subjects have died and we are trying to secure tissue release forms signed by next-of-kin. Sixty-three subjects have refused participation.

We have given 474 (230 African-American and 238 Latinos) tissue release request forms to date to the Tissue Procurement Core Resource at USC/Norris Comprehensive Cancer Center. We are in the process of requesting tissue from 57 additional subjects. We have received tissue on 163 African-Americans and 163 Latinos. All but 14 have been forwarded to Dr. Richard Cote's lab. One hundred fourteen tissue samples have been forwarded to Dr. Gerhard Coetzee's lab and 87 tissue samples have been forwarded to Dr. Juergen Reichardt's lab.

We have received 112 tissue samples from the University of Hawaii; 74 from Caucasians and 38 from Japanese-Americans. All but 10 have been forwarded to Dr. Richard Cote's lab and will then be distributed to Dr. Coetzee and Dr. Reichardt. Thus we have received 438 tissues in all, and we are nearing our final projected target figure.

KEY RESEARCH ACCOMPLISHMENTS

None: This is a Core resource to support the three Projects.

REPORTABLE OUTCOMES

None

CONCLUSIONS

This Core is now functioning very effectively. Prostate cancer patients are being routinely collected among all four racial ethnic groups in this study and signed tissue releases are being routinely obtained. After delays due to IRB issues, tissue procurement and processing is going extremely well at both sites.

REFERENCES

None

APPENDICES

None