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Investigation into the use of antibody phage display to isolate monoclonal antibodies against novel prostate cancer antigens has been conducted. Appropriate materials, which include prostate cancer (PCa) cell lines, tissue specimens from radical prostacetomies, and hybridomas producing antibodies specific for prostatic antigens have been procured. Reactivity of the PCa samples with the antibodies has been tested, and hybridoma mRNA and cDNA has been isolated. . Additional funding and personnel to extend the proposed research has been obtained. Several collaborative arrangements that bring diverse expertise to bear on prostate cancer research problems have been established. These interactions have lead to the development of a nucleus of researchers interested in prostate cancer in African Americans.

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[signature] Feb 29 2000

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Development of Methods to Isolate Recombinant Antibodies Against Prostate Cancer Antigens

INTRODUCTION:

We proposed to develop methods for isolation of monoclonal antibodies (MAbs) against novel tumor antigens by applying phage-display. It has been our intent to produce, in *Escherichia coli,* recombinant derivatives of previously characterized anti-prostate cancer MAbs. These will be used to devise selection techniques that may be applied to direct isolation of antibodies that recognize antigens unique to prostate cancer cells. This project is being undertaken because of the increasing importance of prostate cancer in mortality of American men. In particular, the dramatically higher incidence of prostate cancer among African Americans has not been extensively addressed. Elevated levels of prostate specific antigen (PSA) is the most common biochemical correlate for prostatic cancer used in the clinic. PSA is one of the more useful tumor markers available; however PSA levels are not able to distinguish benign prostatic hyperplasia from malignant carcinoma (Schelhammer & Wright 1993). Furthermore, serum PSA appears to be higher in age-matched studies of African American men as compared with white men (Eastham et al. 1998). There is consequently the need for MAbs that detect additional tumor markers. This need is especially pressing for comparative studies of prostate tumors from black and white patients. Hybridoma-based approaches intrinsically result in isolation of MAbs that bind to both tumor and normal cells. We will replace the process of hybridoma screening by a combination of selective and subtractive procedures using antibody phage display. In this method, the binding domains of antibodies (VH + VL) are displayed on the surface of bacteriophage M13 particles that carry the genes encoding the antibody. This work represents the initial investigation on prostate cancer undertaken by the PI, and was mentored by experienced urologist, Dr. John Petros.

BODY:

Milestones of the investigation that were described in the proposal are listed below as the underlined subheadings for the this section.

Meet with collaborating established investigator.

Several meetings, both at Emory University Medical School and at Clark Atlanta University, have been conducted over the past year. Early meetings included John Petros MD, Joan Karr PhD, (a faculty member at Emory) and Virginetta Cannon PhD. These meetings involved lively exchanges regarding important research problems and how the investigators technology can best address them. Whereas our original concept proposed using Dunning rat prostate cell lines for our selection procedure, our ultimate target is human disease. Rodent cells have been demonstrated to express different antigens, so it was decided that human PCa cell lines are more appropriate for antigen-based selection. We obtained human PCa cell lines PC3, DU-145 and LNCaP from Dr. Petros' technical associate, Ms. Carrie Sung. It was further decided that LNCaP cells (Horoszewicz *et al*

1980) would be the target line of choice, since unlike the other 2 human lines, it has characteristics of the kind of transformed cells we will ultimately be seeking to screen. LNCaP cells are androgen-sensitive and express well characterized prostate epithelial biomarkers prostate specific antigen (PSA) and prostate specific membrane antigen (PSMA). According to published reports, neither DU-145 nor PC3 cell express either antigen and are not stimulated by androgen. In our hands however, these cell lines are easier to grow than LNCaP's and they are being maintained for particular classes of experiments.

Purchase hybridomas from ATCC
Isolate RNA from hybridoma samples
Generate cDNA and PCR amplify immunoglobulin genes

Several hybridoma cell lines were purchased from the American Type Culture Collection. However, because of high turnover of the students and technicians who were responsible for maintenance of the cultures, our stocks were contaminated and had to be repurchased on more than one occasion. After several trials, we have been able to grow four lines (F5-A-1, 7E11-C5, 9H10-A4, and RLSD09). Hybridoma and monoclonal antibody designated 7E11-C5 (7E11) is reported to show crossreactivity to both normal and neoplastic prostatic epithelium. This widely used antibody identifies an 100 kDa glycoprotein known as Prostate-Specific Membrane Antigen (PSMA). This integral membrane protein may be a surface peptidase, is expressed in prostate epithelial cells and is downregulated by androgen (Fair *et al* 1997). 7E11 derivatives have been used to image nodal and metastatic prostate cancer in humans (Chengazi *et al* 1997). Hybridoma and monoclonal antibody designated 9H10-A4 (9H10), is an antibody specific for LNCaP cells but further characterization of its antigen has not been reported (Horoszewicz, *et al.* 1987). RLSD09 is directed against a "33kDa glycoprotein antigen from human prostate" which is identical to Prostate Specific Antigen (PSA; Loor *et al.* 1987). Finally, hybridoma and monoclonal antibody designated F5-A-1/22.8.13 (F5) is specific for PSA (Papsidero et al. 1885).

Immunoglubulin was detected in culture supernatants of all four hybridoma lines; we were able to determine however that 7E11-C5 is of IgG1/κ rather than IgG2a as was indicated in the materials supplied from the ATCC. The amount of antibody varied from culture to culture and according to the age of the cultures. We obtained ~10^7 cells for each of 3 lines (F5, 7E11 and RLSD09) at a stage when antibody production is high, and have prepared total RNA and cDNA from each. We are continuing to perform procedures for molecular cloning of the antibodies, including PCR amplification of immunoglobulin cDNA, ligation of the genes into phage-display expression vectors, and phage selection on their cognate antigens.

Obtain hybridomas from other investigators

Our requests to two other investigators for prostate-specific hybridomas have resulted in either no response, or the assertion that "it will not work in your system". This avenue has not been pursued further, but has not been excluded as an option.

Grow up hybridomas and test culture supernatants against PCa antigens

We extensively tested our hybridoma culture supernatants against cultured PCa cells and lysates. Enzyme linked immunosorbent assays (ELISA) on whole cells were performed according to the method of Rochon *et al* (1994). Briefly, cells were grown to confluence, harvested in PBS, and resuspended at 1.5 x 10^7 cells/mL in 3mL of 3mM Tris.HCl (pH 7.4), 0.3mM MgCl2 and 0.5 mM PMSF. Twenty µL of cell suspension (3 x 10^5 cells) were added to the center of a well (96-well flat bottom Immulon, Dynatech) and dried at 37°C. Once dry, the cells were fixed for 30 min at RT with 2% formaldehyde (pH 7.4), and washed 3 times with PBS, and the plates were blocked for 2 hr at RT with 3% bovine serum albumen (BSA, Sigma) in TBS + 0.5% Na azide. When necessary plates were stored at 4°, and warmed to 37° prior to use.

PCa cell lysates were prepared by sonication. Approximately 10 -20 x 10^6 viable cells of cells/mL in Hanks' balanced salt solution (GIBCO) containing 2mM PMSF and 20µL/mL of trasylol were sonicated for 2.5 min to disrupt the cells into fragments. This lysate, equivalent to 5 x 10^4 cells/70µL were added to each well of a microtiter plate (Dynatech) and the plate was centrifuged for 3 min at 1400 x g on a Sorval T6000 centrifuge at 20°. 1% formaldehyde in TBS was added and the lysates were allowed to fix to the well for 15 min at RT. The wells were then rinsed 3 times with TBS and filled with 3% bovine serum albumen (BSA, Sigma) in TBS + 0.5% Na azide.

Hybridoma supernatants were applied undiluted, incubated for 1 hr at RT, the wells washed, and probed with Goat anti-mouse K-alkaline phosphatase, Goat anti-mouse IgG1-AP or Goat anti-mouse IgM-AP. Color development of ONPP substrate was monitored visually and measured on a microplate reader.

All supernatants tested showed specific reactivity with whole LNCaP cells. Reactivity with cell lysates was significant for 7E11-C5, but did not exceed background levels for RLSD09 (Figure 1) and 9H10. No detectable reactivity with PC3 cells was seen.

The crude sonicates of LNCaP cells were also run on reducing SDS-PAGE, transferred to nitrocellulose membranes and immunostained with culture supernatants and the appropriate AP-conjugated secondary Ab. No reactivity of RLSD09 was seen, though PSA could be detected using commercial Rabbit-anti-PSA (Figure 1). This observation suggests that RLSD09 does not recognize SDS-denatured PSA. In contrast, 7E11 strongly identified a ~ 100 kD band, corresponding to PSMA. To our surprise, F5 showed a major band at ~ 80 kDa with minor bands at higher molecular weight. The published data on this MAb imply that it bind to PSA, and we know that PSA is present in these lysates. Our results are thus difficult to reconcile with previously reported observations. ELISA using this MAb against commercial preparations of PSA also yield negative results. We anticipate further characterization of the specificity of this MAb, which clearly binds to a LNCaP antigen, once we have cloned and expressed the gene in *E. coli*. A weak ~ 120 kD band was seen with 9H10 (data not shown).

Provide active culture supernatants to collaborator (VC) for immunocytological analysis

Immunofluorescence microscopy was performed on samples of radical prostectomies from white and black patients, with the objective to correlate antigen expression and ethnic origin of these tissues. The studies described below were performed in the laboratory of Dr. Virginetta S. Cannon, Assistant Professor of Biology Morehouse College, and laser confocal microscopic analysis was performed at Clark Atlanta University. The results of which may provide new and potentially valuable information regarding phenotypic differences and similarities among prostate cancer antigens from black and white patients.

The relative amounts of cancerous and normal tissues were initially assessed using standard histological techniques of hematoxylin and eosin staining. Prostatectomy sections were processed by standard means, namely, fixed in10% neutral formalin, embedded in paraffin and cut into 4-mm sections. The tissues were deparaffinized with two changes of xylene, then rehydrated with a descending ethanol series. Tissue sections were stained 30 seconds in hematoxylin, washed several times, partially dehydrated, then stained 2-3 minutes in eosine. Following ethanol washes to remove excess eosine staining, the sections were dehydrated and coverslipped using a xylene-based mounting medium. Results of staining showed blue nuclei and pink cytoplasm.

Normal epithelia were identified by their characteristic two cell layers and large complex glandular architecture. Hyperplastic (pre-cancerous) areas, also called prostatic intraepithelial neoplasia (PIN) were identified by their characteristic multi-cell layered epithelia with preservation of basal cell layer. Prostate cancer is identified at low magnification by the architectural appearance of small, closely packed glands and confirmed at high magnification showing loss of the basal cell layer, large nuclei and prominent nucleoli. (Figure 2).

In order to correlate the observed hyperplastic areas with specific prostate antigen expression a number of monoclonal antibodies specific to prostate specific antigen (PSA), and prostate specific membrane antigen (PSMA) were employed. Briefly, sections were deparaffinized in xylene and rehydrated in graded ethanol concentrations (100% to 50%). Nonspecific binding of secondary antibody was blocked by preincubating the sections with species specific preimmune serum. The sections were then incubated for 2-4 hours in the primary monoclonal antibody, followed by incubation with the Texas red-conjugated secondary antibody for 30 minutes to one hour, mounted with coverslips and examined using Confocal Laser Microscopy. In general, the amount of antigen present was quite variable. RLSD09 gave the most intense staining pattern (see Figure 1B). It is noted however that this antibody recognizes both normal and malignant tissue antigens. It would seem that localization of RLSD09 (PSA) in areas of architectural disturbances would indicate localized prostate cancer antigens(Figure 3a). In the lower left of the image shown, there is heavy staining of PSA in a gland, that is nearly invisible under phase contrast. Since PSA staining has been shown to be reduced in high grade prostatic tumors, this may represent a glancing section of a normal gland, or a tumor that has not undergone significant dedifferentiation. Staining with 7E11 was not as intense, but did similarly localize in the epithelium (Figure 3b). Very little antigen

could be found using F5 and 9H10 antibodies, although these showed to have high titers in culture supernatants. The reason for this is unclear. Although 9H10 was raised against LNCaP tumor cells, its expression was not commonly seen in biopsy specimens (Horoszewicz, *et al.* 1987).

To date we have examine specimens from only one black and two white individuals. No striking differences have been observed between these samples. Obviously methods that allow high throughput analysis of large populations of specimens are required. We are investigating multiple color and digital imaging methods which together with quantitative examination might provide noteworthy correlations.

As indicated above, although we are well on our way, we have not yet succeeded in obtaining molecular clones of the anti-prostatic antigen MAbs. We nevertheless plan to continue the proposed work as well as develop other areas of research on prostatic carcinoma. Strong collaborative interactions have been developed with researchers at Emory University and with scientists at the Atlanta University center.

A number of possible phage selection procedures for recombinant antibody-phage (PhaBs) have been discussed and investigated. Pathologists Mulal Amin MD and Andrew Young MD in the Winship Cancer Center at Emory have agreed to make their laser capture microscope available for possible selection of PhaBs binding to slides of fixed prostate tissue. Such technology would allow dissection of cancerous tissue out from sections containing both normal cells, which would serve to subtract PhAbs specific for normal antigens, and cancerous cells. (Figure 4).

The PI has been actively involved in the establishment of the Prostate Cancer Alliance (PCA). This group exists to further the diagnosis, treatment, and ultimately a cure for prostate cancer. It is a multidisciplinary investigational team with an emphasis on minority health. The PCA was founded and is centered at the Atlanta University Center and is comprised of researchers from academic and industrial institutions including Boston University, Clark Atlanta University, D-squared Technologies, Inc., Meharry Medical College, Morehouse College, Oncotech, Inc., Spelman College, and Tuskegee University. A student who was supported by this USAMRMC MPFT training award spent several weeks this summer learning tissue culture and immunoblotting methods in the lab of Duane Johnson at Morehouse College. As a result of this collaboration, a short talk and a poster presentation may be credited to USAMRMC support (see REPORTABLE OUTCOMES, APPENDIX A). These studies detail immunochemical analysis of cycloxygenase expression in LNCaP cells following treatment with potential therapeutic agents. Extensive examination of PSA & PSMA expression in following these treatments has not yet been performed.

Develop proposal idea for a more expansive project

As a consequence of discussion with the experienced investigator (Dr. Petros) and other collaborators, a proposal was submitted as part of the NIH/RCMI (Research Centers in Minority Institutions) for Clark Atlanta University. This project was funded, and will provide partial salary support for Dr. Williams, Dr. Petros, and a postdoctoral associate who has yet to be identified. We are currently developing additional ideas into

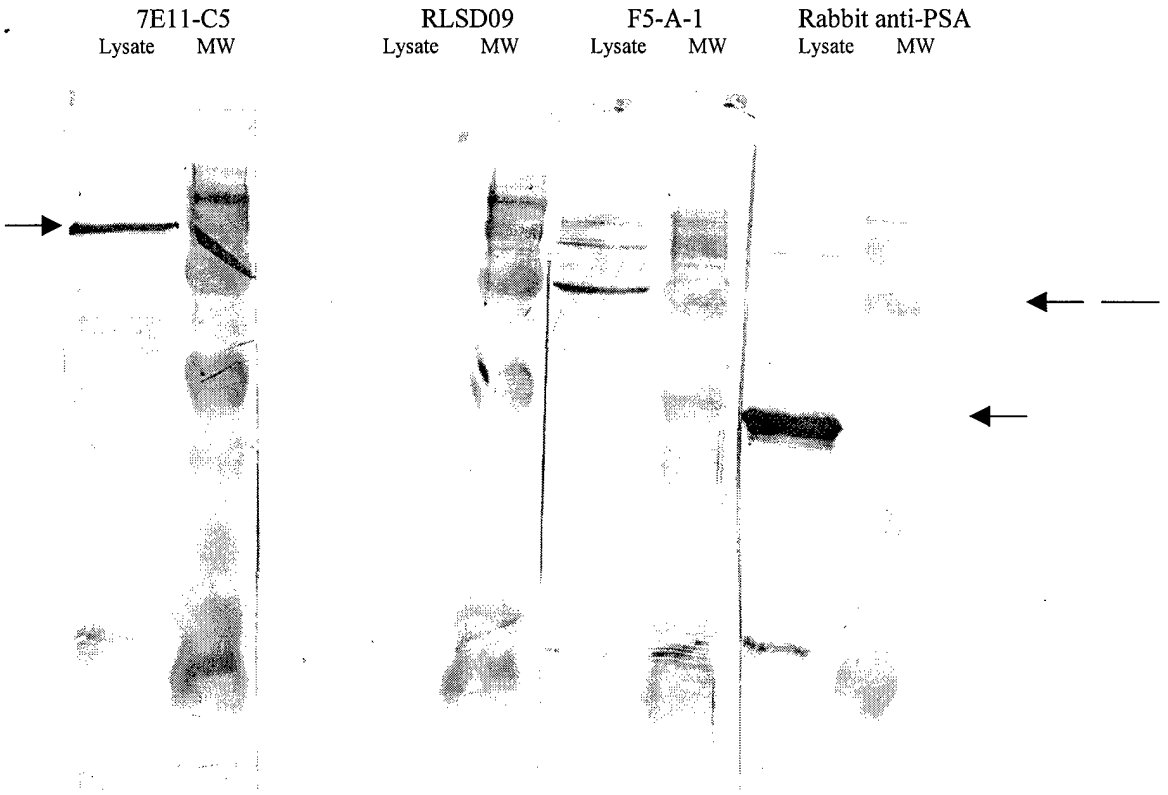


Figure 1: Western blot of LNCaP cell lysates probed with monoclonal antibodies 7E11, RLSD09 and F5. BioRad Kaleidoscope molecular weight standards are included next to each lysate sample.

Figure 2

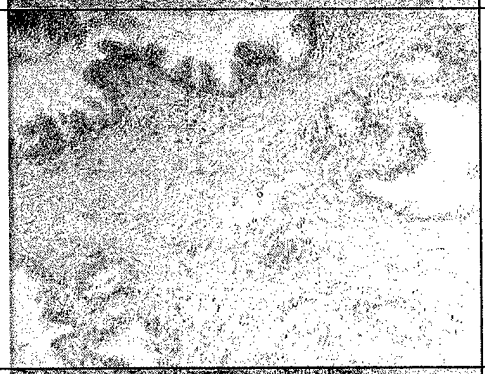
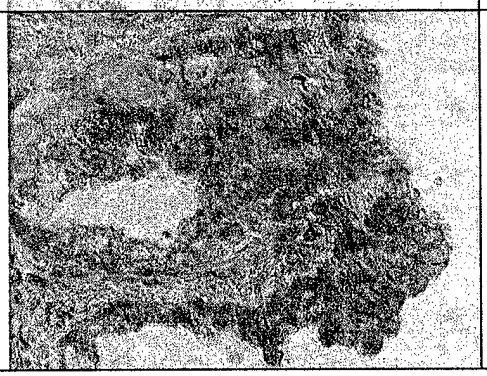
H&E stain Low magnification showing complex glandular architecture		
High magnification of glands with prostatic intraepithelial neoplasia (PIN)		
Section showing normal epithelia, smooth muscle and connective tissues		
Cancerous gland		

Figure 3

Immunofluorescence microscopy of sections from radical prostactomies, probed with monoclonal antibodies.

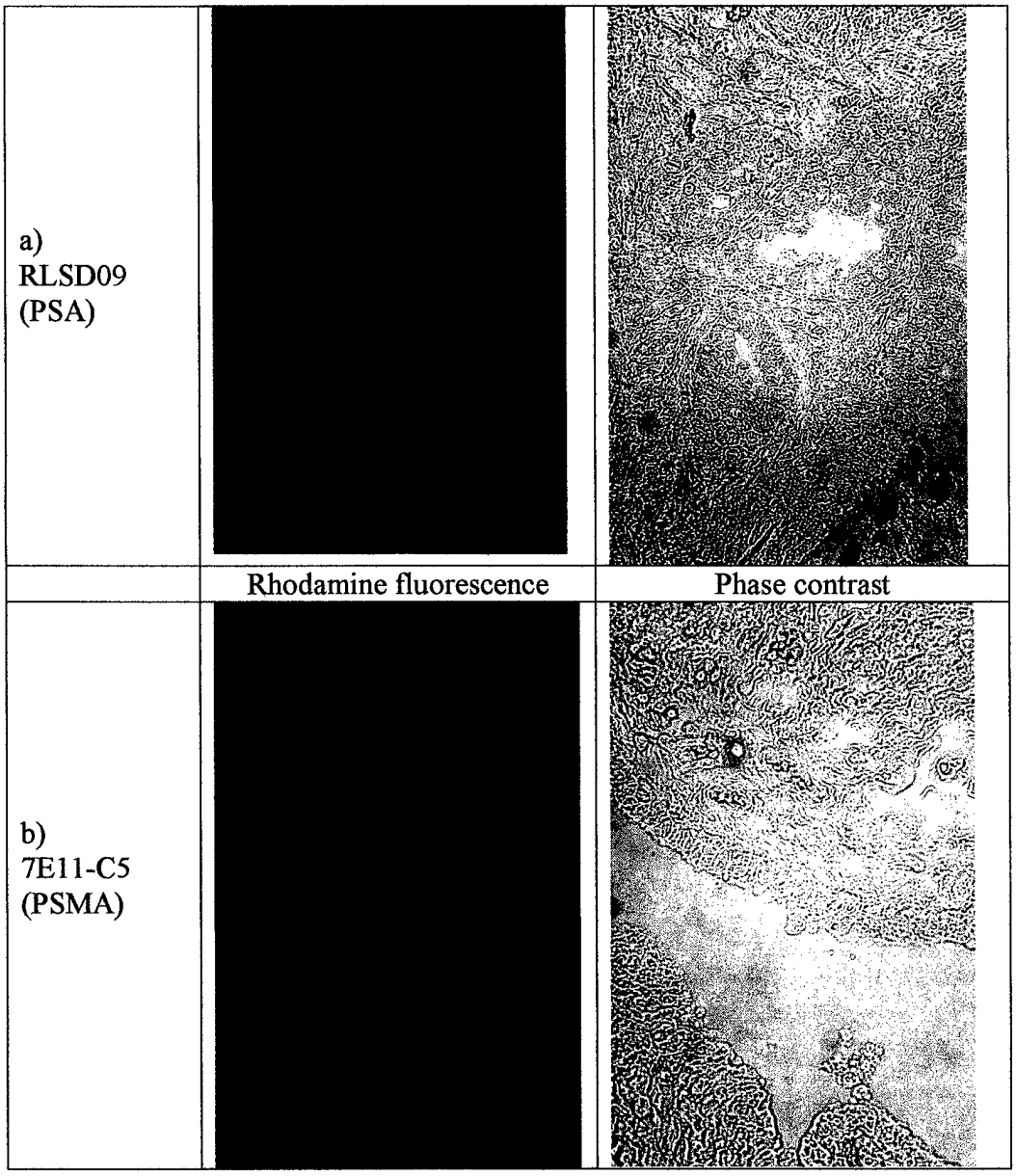
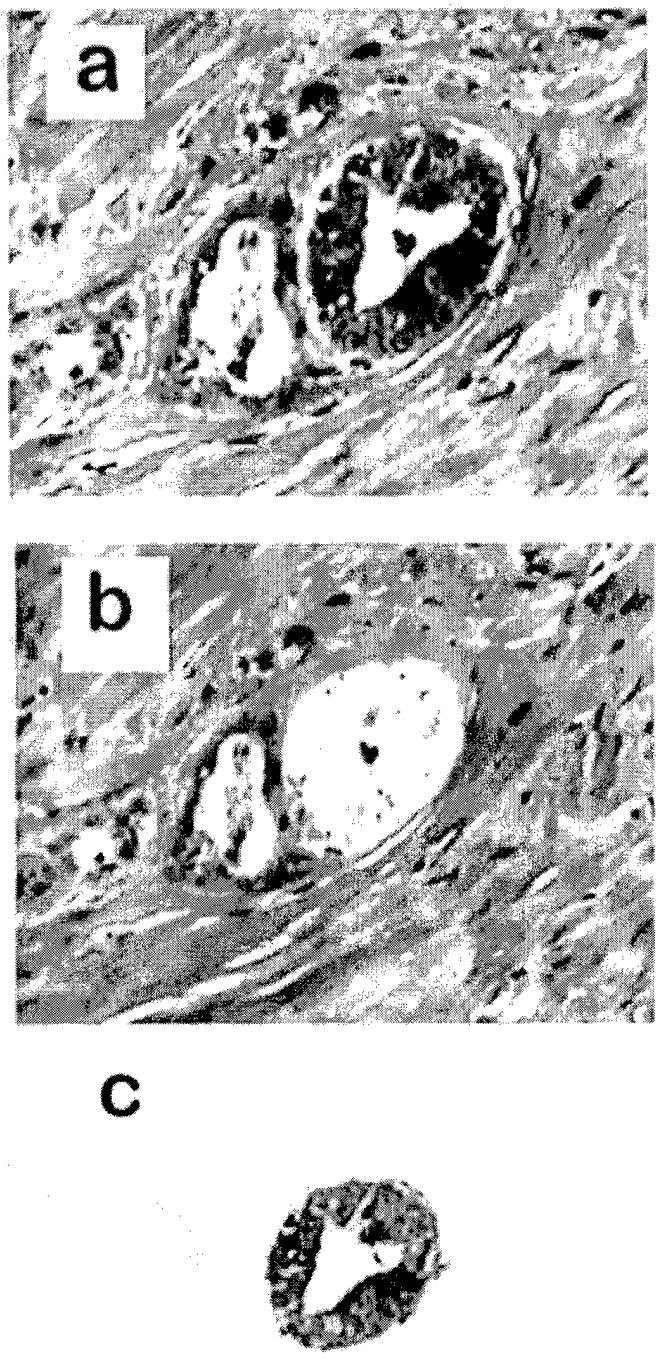


Figure 4. Laser capture excision of tumor from prostate tissue sections (*courtesy of Andrew Young MD*)
a - cancerous gland
b - remaining tissue following excision
c- excised tumor



a proposal to be submitted in response to a program announcement from the National Cancer Institute (Applications Of Innovative Technologies For The Molecular Analysis Of Cancer: Phased Technology Application Award [R21/33]).

KEY RESEARCH ACCOMPLISHMENTS:

- First steps have been made towards the utilization of a very promising technology (antibody-phage display) for identification of novel cancer antigens
- Collaborations have been established with a variety of clinical and basic research laboratories
- Immunocytochemical studies have been conducted on radical prostatectomy specimens
- Our laboratory is now committed to prostate cancer research

REPORTABLE OUTCOMES::

➢ Presentation:

"Novel isochlacone compounds induce down-regulation of COX-1 in human metastatic prostate cancer cell line LNCaP". K.P. Johnson, G. Rowe, D.B. Cooke, P. Campbell, M. Williams, G. Charles, Q. Matthews, B.A. Jackson and D.E. Johnson. Platform presentation at the Research Symposium of the RIMI Program, Atlanta GA, Feb 18-21, 2000 AND poster to be presented at the 91st Annual Meeting of the American Cancer Society, April 1-5, 2000.

See abstract in Appendix A

➢ Funding Applied for Based on Work Supported by This Award

NIH/RCMI subproject grant "Recombinant Antibodies against Prostate Cancer Antigens", Clark Atlanta University RCMI, received Oct 1999.

NIH "Applications Of Innovative Technologies For The Molecular Analysis Of Cancer: Phased Technology Application Award (R21/33)". Application pending July 2000.

➢ Employment or Research Opportunities

Students receiving training and support under the auspices of this award include Ms Quiana Matthews, Ms. Candice Flowe and Ms. Onoh Oboh.

- Ms. Matthews was supported in summer of 1999 and has gone on to a graduate program at Meharry Medical School.
- Ms. Flowe is a graduate student in the Department of Biological Sciences at CAU and is currently the lead person on continuation of the project. She received tuition support in Fall of 1999.

- Ms. Oboh is an undergraduate Biology major who is currently assisting us with cell culture and laboratory maintenance.

CONCLUSIONS:

The aims of the Minority Population Focused Collaborative Training awards are the elaboration of a training plan and concept development. We are have developed a concept for the identification of novel biomarker antigens with the intent of focusing on the disparity in prostate cancer incidence and mortality between African Americans and other ethnic groups. Collaborations have been established with a urologist, pathologists and a group of basic scientists for the purpose of further investigating the characteristics, diagnosis and treatment of prostate cancer in black men. Sources of test material have been identified, methods for immunochemical analysis of samples have been worked out, and a protocol for the use of new technology (phage display) have been established. Several of our initial concepts have been revised based on experimental evidence. For example, the isotypes and antigen specificity of established hybroidomas, knowledge of which is essential for the molecular cloning and selection procedures to follow, have been called into question, and established for the material we have available. Similarly, expertise in mammalian cell culture, which was not available in our laboratory at the outset of this project, is incrementally being obtained. Our laboratory is now committed to research on antigens expressed in prostate cancer, and we are well positioned to bring our resources to bear on this problem towards the amelioration of the lives of all Americans.

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APPENDIX A:

NOTE: ABSTRACT O-26 IS IN SEQUENTIAL ORDER.

Infrastructure Building, Research Productivity and Collaborations Keys to Success in the New Millennium

Research Symposium and Program Directors Meeting

February 18 - 21, 2000
Ritz-Carlton Hotel
Downtown, Atlanta, Georgia

Hosted by: Spelman College
National Institutes of Health
National Center for Research Resources

O-16

NOVEL ISOCHALCONE COMPOUNDS INDUCE DOWN-REGULATION OF COX-1 IN HUMAN METASTATIC PROSTATE CANCER CELL LINE LNCAP. K.P. Johnson, G. Rowe, D. B. Cooke, P.E. Campbell, M. Williams, G. Charles, Q. Matthews, B. A. Jackson, and D. E. Johnson. Clark Atlanta University, Boston University, and Morehouse College.

Cyclooxyegenase (COX-1 and COX-2) enzymes are the rate-limiting step in prostaglandin synthesis. There have been recent studies that indicate relative changes in these COX enzyme levels in different types of carcinomas. Also, COX enzymes have been induced by mitogenic growth factors such as epidermal growth factor (EGF). Therefore, our laboratory examined the role of COX enzymes and EGF in metastatic prostate cancer. This role was examined by the use of Western blot analysis in human metastatic prostate cancer cell line LNCaP (androgen dependent). LNCaP cells were treated with novel agents DJ52, DJ53, and known anti-BPH agent finasteride; they were then evaluated for their expressions of COX-1 and EGF. LNCaP cells were treated with the three agents at concentrations of 10^{-6}M, 10^{-5}M, and 10^{-4}M and compared with untreated cells (control). COX-1 and EGF expression in LNCaP cells were compared to LNCaP growth inhibition data. The results indicated a down-regulation of COX-1 levels at 10^{-6}M and 10^{-4}M. Finasteride demonstrated no significant change in COX-1 levels at any of the tested concentrations of DJ52. DJ53 induced a concentration-dependent decrease of COX-1 protein levels in LNCaP cells. DJ52 demonstrated a decrease in EGF protein levels at 10^{-6}M, but showed no significant decrease in EGF at concentrations of 10^{-5}M and 10^{-4}M. DJ53 and finasteride demonstrated a concentration-dependent decrease in EGF protein levels. These data indicated a direct relationship between EGF and COX-1 protein level changes as the growth of LNCaP cells was inhibited. Therefore, itappears that EGF and COX-1 plays an important role in cellular proliferation of human metastatic prostate cancer cell line LNCaP.