AD________________

Award Number: MIPR8LBN638928

TITLE: Fatty Acid Binding Proteins in Prostate Cancer

PRINCIPAL INVESTIGATOR: Marti Jett, Ph.D.
Fred Hawkins

CONTRACTING ORGANIZATION: Walter Reed Army Institute of Research
Washington, DC 20307-5100

REPORT DATE: January 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

DTIC QUALITY INSPECTED 4

20001213 113

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 2000	Annual (1998 - 1999)

4. TITLE AND SUBTITLE
Fatty Acid Binding Proteins in Prostate Cancer

5. FUNDING NUMBERS
MIPR8LBN638928

6. AUTHOR(S)
Marti Jett, Ph.D., Fred Hawkins

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Walter Reed Army Institute of Research
Washington, DC 20307-5100

E-MAIL:
Marti.Jett@na.amedd.army.mil

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

We have shown that there is a distinct pattern of fatty acid binding protein (FABP) expression in prostate cancer vs normal cells and that finding has be confirmed in patient samples of biopsy specimens. Of 6 different FABP studied, certain FABP were identified as markers that may be exploited to reveal stage or aggressiveness of prostate cancer. Since FABP are secreted from cells, we have shown that liver FABP was found in the extracellular fluid of prostate cancer but not prostate normal cells and this characteristic may provide an opportunity to use FABP patterns in body fluids as markers for prostate cancer and stage specific screening. Transfection of adipocyte (A)-FABP into prostate cancer cells (that lack A-, but have abundance of L-, and Intestine (I)-FABP) caused the cells to become more normal-like; they showed decreased proliferation and induction of genes involved in apoptosis. Similar results were seen upon using antisense to L-FABP to accomplish the same end results. Those findings indicate that FABP are important in prostate cancer cell regulation. Heteropolyanions show good potential for therapeutic intervention in prostate cancer and key experiments are underway to verify those results *in vivo*.

14. SUBJECT TERMS
Prostate, fatty acid binding proteins, detection, apoptosis, bioactive lipids

15. NUMBER OF PAGES
43 including Appendix

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

3-23-00

PI - Signature Date

TABLE OF CONTENTS

FATTY ACID BINDING PROTEINS AS A POTENTIAL MARKERS AND THERAPEUTIC TARGETS IN PROSTATE CANCER

(1) Front Cover..1

(2) SF 298..2

(3) Foreword..3

(4) Table of Contents..4

(5) Introduction..5

(6) Body..5

- **a) Background..................................5**
- **b) Statement of Work Task 1 correlated with accomplishments...........7**
- **c) Statement of Work Task 2 correlated with accomplishments...........8**
- **d) Statement of Work Task 3 correlated with accomplishments..........13**
- **e) Statement of work showing what objectives are completed, which did not work, etc. ..15**

(7) Key Research Accomplishments...................................16

(8) Reportable Outcomes..,.16

- **a) Bibliography: Manuscripts, abstracts, patents...16**
- **b) Development of cell lines.............................17**
- **c) Animal models used..................................17**
- **d) Personnel receiving pay from this grant...........17**

(9) Conclusions..17

(10) References..17

(11) Appendices..21

(5) INTRODUCTION

The exact role of fat metabolism and bioactive lipids in prostate cancer is unclear, however, numerous reports show an involvement of dietary fat with increased incidence of prostate cancer an also prostate cancer tissue vs. normal tissue has been characterized as having an unusually high production of bioactive lipids. Therefore, we proposed that the family of proteins (Fatty acid binding proteins, FABP) involved in transport, utilization and regulation of lipid metabolism would likely play an important role in prostate cancer. The purpose of this study is to (a) examine the pattern of expression of FABP in prostate normal and cancer cells, tissues, and extracellular fluids and (b) determine the role of the various classes of FABP in regulation of cellular function and (c) delineate the relationship of growth factors with FABPs.

The scope of this study relates to understanding the role and regulation of FABP in prostate normal and cancer cells so that this family of proteins can be utilized (a) in rapid detection and staging of aggressiveness of prostate cancer and (b) for therapeutic targets.

(6) BODY

a) Background

There is evidence that high dietary fat intake may be a contributing factor in the initiation or development of prostate cancer. Arachidonic acid, a potent bioactive lipid, has been shown to be significantly higher in prostate tumor vs normal cells. The family of cytoplasmic proteins, known as fatty acid binding proteins (FABPs), are crucial transporters of bioactive lipids, which, in turn, continuously stimulate proliferation (5, 16, 17, 35, 36, 38, 69). Because they bind bioactive lipids which initiate signals that result in increased mitogenesis, FABPs might play a crucial role in promotion of prostate cancer cell growth. The expression of one of the FABPs, designated L-FABP, has been shown to be upregulated in liver during carcinogenesis (16, 17, 61). Only one previous study addressed the issue of FABPs in prostate cancer (13) and it suggested that an L-FABP -like protein occurred in prostate cells. Other FABP types have been implicated in control of cell proliferation or cancer (11, 20, 33, 46). We have examined 6 different types (there are 12 related types of FABP, see Current Research section below) of FABPs and have found their expression to be altered in prostate cancer vs normal cells. We will test the hypothesis that prostate cells express more than one type of FABP and that the relative changes in the levels of these FABPs might play a major role in the tumorigenesis of the prostate gland. Key questions to be answered include:

TASK 1. Characterize patterns of FABP expression in prostate normal/tumor cells, tissues, extracellular fluids.
i) Of six FABP types associated either with promotion or restriction of cell proliferation or cancer, are there differences in patterns/ratios of their levels in a battery of prostate cancer cells (from different stages of progression) compared to the normal cells? Are similar differences also detectable in normal/tumor pairs of graded human biopsy samples?
ii) Are these FABPs secreted into cell culture media? Can they be identified in plasma or other body fluids during carcinogenesis of the prostate gland and correlated with PSA levels and pathological stage analysis?

TASK 2. Determine relationship of FABP with cellular regulatory processes. This task addresses the question, "Are FABP really important in prostate cancer?".
i) Can transfection of the FABP genes into prostate normal cells change the tumorigenicity of these cells, *in vitro*? Can we convert an aggressive prostate cancer cell into a non-tumorigenic line by changing the balance of these different FABPs *in vitro*? Can tumor induction in a mouse model be altered by blocking gene expression of certain FABP(s)?
ii) Are the expression levels of these FABPs altered by exogenous agents such as androgens, growth factors, fatty acids, bioactive lipids, perixosome proliferators, antisense oligonucleotides, FABP peptides or drugs specific for these proteins?

TASK 3. Heteropolyanion (HPA) drugs and their potential usefulness for treatment of prostate cancer.
i) Examine HPA ability to block proliferation in prostate cancer cells in vitro as well as xenografts of these cells in nude or SCID mice.

ii) Determine the effects of HPA on expression patterns of FABPs

Epidemiological studies on carcinoma of the prostate gland have shown a positive relationship between the consumption of dietary fats and development of prostate cancer (22, 57). This led to the suggestion that high dietary fat intake may be a contributing factor in the initiation or development of this tumor (73). Conversely, Wang et al. (71) showed that lowering the quantity of fat as a proportion of total calories decreased the growth rate of human prostate adenocarcinoma cells in mice.

Studies have implicated an association of linoleic acid (LA) with prostate cancer. Linoleic acid is the most prevalent unsaturated fatty acid component of commonly used cooking oils. A large prospective study of American men showed a positive association between linoleic acid in the diet and prostate cancer (24). Moreover, *in vitro* studies of the human prostate cancer cell line PC-3 showed stimulated growth in the presence of linoleic acid whereas the long chain fatty acids may inhibit tumorigenesis (52). Recently Harvei et al (29) showed an association of serum levels of linoleic acid and palmitic acid with increased risk of prostate cancer.

Fat-derived arachidonic acid metabolites are potent mitogens:

A potential mechanism to account for the relation between linoleic acid intake and prostate cancer derives from the fact that linoleic acid serves as a precursor for arachidonic acid (AA). Depending on the tissue in question, phospholipids contain high quantities of AA at the Sn-2 position. AA is the most important fatty acid of the cell membrane since it serves as a precursor for eicosanoids such as prostaglandins, HETEs and leukotrienes. These latter molecules function as local hormones and modulators of cell function and thus are members of a potent group of bioactive lipids.

There are multiple reports supporting a role for AA in the proliferation of prostate cancer cells. In radical prostatectomy specimens, AA turnover was 10 times higher in the tumor as compared to surrounding uninvolved prostate tissue. Diets high in corn oil, a fat rich in the AA precursor linoleic acid, markedly stimulated the growth of human prostate cancer xenografts in nude mice (71). AA has been also reported by Ghosh and Myers (23) to be an effective growth stimulator of prostate cancer cells. We have shown that 5-HETE directly stimulated proliferation in small cell lung carcinoma cultures (2) and blockers of 5-lipoxygenase (LO) pathway induced apoptosis and altered eicosanoid generation. Selective blockade of the different metabolic pathways of AA revealed that the growth stimulatory effect of AA was inhibited by 5-lipoxygenase specific inhibitors AA861 and MK886. We found that addition of 5-HETE showed stimulation of lung and breast cancer cell growth similar to that of IGF-1, whereas the leukotrienes were ineffective. Chaudry et al (12, 14) reported that concentrations of AA and docosapentaenoic acid are significantly lower in malignant prostatic tissue as compared to benign prostatic tissue. The reduction in AA was thought to be primarily the result of increased utilization of this fatty acid resulting in higher concentrations of its metabolites. Furthermore, it has been shown that human prostate cancer cells rapidly undergo apoptosis when they are deprived of AA or when the subsequent metabolism of AA by the 5-LO pathway was interrupted (23). These data provide a mechanism by which dietary fat intake might influence the natural history of prostate cancer through its effect on arachidonic acid metabolism.

Fatty acid binding protein involvement in utilization/trafficking of mitogenic lipids:

Intracellular transport /utilization of bioactive lipids is a critical component in the process by which these molecules continuously stimulate proliferation through interactions with nuclear receptors. Transport and utilization have been proposed to be mediated by an important family of cytoplasmic proteins known as fatty acid binding proteins (FABPs) (59). These proteins may also be important in the regulation of free fatty acid concentrations within the cell. Such crucial participation in the trafficking and availability of fatty acids suggests that the FABPs may be critical links in the mechanistic chain connecting dietary fat with cancer.

FABPs are found in abundance in a variety of tissues. The members of this broad multigene family currently consist of at least seven types whose amino acid sequences have been obtained from protein purified from tissue or from cDNA nucleotide sequences from tissue RNA (6, 30, 42, 44, 48, 56, 62) The designations for each of the FABPs is derived from the human tissue from which it was isolated and includes: 1) adipocyte (A-FABP), 2) heart or muscle (H-FABP), 3) brain (B-FABP), 4) epidermis or psoriasis-associated (E-FABP), 5) liver (L-FABP), 6) intestine (I-FABP), and 7) myelin or P2 (P2-FABP). A frequently studied FABP from bovine mammary gland, designated MDGI (mammary-derived growth inhibitor) and thought to be a distinct type in itself, was later determined to be H-FABP (40, 60).

Expression of each FABP type is not necessarily limited to the tissue from which it was originally isolated. In some tissues FABP expression is developmentally regulated and different types may be expressed in different regions of an organ.

The properties common to FABPs include their intracellular abundance, their small size (a molecular weight range of 14-16 kDa and an average of 132 amino acids), their sequence relatedness and three-dimensional structure and their ability to bind a variety of lipids. Closely related in structure to these FABPs are the two cytosolic retinol binding proteins (CRBP I and II), the two cytosolic retinoic acid binding proteins (CRABP I and II) and ileal lipid binding protein (ILBP) (1, 15, 21, 41, 45). As a group A-FABP, H-FABP, B-FABP, and E-FABP in humans share between 50-65% protein sequence homology and contain a tyrosine near residue 20 that can be phosphorylated. These four FABP share only 20-25% homology with L-FABP or I-FABP which do not have the tyrosine. L-FABP is distinguished by its lack of the amino acid tryptophan. The crystal and solution structures have been determined for several of the FABPs with or without bound lipid and all reveal a common structure of a barrel shape formed from the interaction of 10 β strands with the internal core serving as the lipid binding site [reviewed in (4)].

Certain FABPs have been reported to have differential effects on cell growth when cDNA clones have been transfected into these cells. Transfection of L-FABP into hepatoma cells increased proliferation (36, 38, 59). In contrast, MDGI (H-FABP) appears only in lactating normal and not tumor mammary cells (26-28) and transfection of a cDNA clone of MDGI into breast cancer cells or mouse mammary epithelial cells results in loss of tumorigenicity (33). FABPs are known to bind many different groups of fatty acids and their derivatives, including eicosanoids and other bioactive lipids [reviewed in (69)]. L-FABP exhibits different lipid binding characteristics from that of A-FABP or H-FABP. L-FABP transfected into rat hepatoma cells also mediates cell induction by carcinogenic peroxisome proliferators (39). Several studies suggest that FABP increases the solubility of fatty acids in the cell cytoplasm causing a net diffusion of fatty acids from the plasma membrane to the intracellular membrane compartments (66, 70).

Changes in expression of FABPs have been reported for bladder cancer. Psoriasis-associated FABP (E-FABP) was noted to increase in level with increase in differentiation of bladder squamous cell carcinomas (46). Although FABPs are intracellular proteins, H-FABP has been detected in elevated levels in plasma and urine of patients suffering from myocardial infarction (58, 67, 72), whereas psoriasis-associated FABP (E-FABP) was among a number of marker proteins detected in the urine of bladder cancer patients (50). In addition, loss of adipocyte-FABP (A-FABP) was reported with progression of human bladder transitional cell carcinomas (11). The presence of A-FABP correlated with the grade and stage of the disease. The A-FABP protein was present in high levels in grade I and II TCCs whereas grade III had 37% reduction and grade IV had no A-FABP expression. A-FABP may act as a growth inhibitor similar to the MDGI (H-FABP) protein in breast cancer and loss of A-FABP expression may serve as a prognostic marker for aggressive bladder cancer. Transfection of a cDNA clone of rat A-FABP into normal rat L6 myoblasts resulted in a two fold increase in their proliferation rate and was suggested to indicate that A-FABP functions similarly to L-FABP (49). However, this study did not address the question of A-FABP's effect on tumor cells.

Significance of the Research: This study will provide the basis for a better understanding of the direct role of fatty acids/bioactive lipids and FABPs in development and progression of prostate cancer. FABPs are potential markers for prostate cancer or, more specifically, for an aggressively growing prostate cancer. Therefore, evaluation of several markers of prostate cancer, together, may help patients choose their treatment modality. FABPs also present a logical target for intervention with therapeutic drugs which can be designed to interrupt their crucial function of transmitting the cascades of signals of bioactive lipids which continually promote cancer cell proliferation.

This study may provide information indicating the potential usefulness of FABPs as possible early detection markers, along with PSA levels, and give some meaningful answers in identifying different stages of prostate cancer.

b) Statement of Work Task 1correlated with accomplishments

The objective of Task 1 in the SOW was to define the relationship of FABP expression levels in prostate normal and tumor cells, tissues and extracellular fluids. This study has been written as a manuscript and submitted to the journal "Cancer Research" for publication and the submitted manuscript is attached as

Item #1 in the Appendix. All references to portions of that manuscript, figures and tables will be indicated as MS#1.

Methods: MS #1 describes design of specific primers (Table 1, MS#1) so that expression of the 6 FABP as well as CRABPI could be measured by RT-PCR as well as by ELISA-PCR. The details for all methods used for this part of the study are described in MS#1. These methods include details about RT-PCR procedures, extraction of RNA from cells and tissues, and detection of L-FABP in culture fluid of prostate cancer but not normal cells.

Accomplishments: The FABP expression profile for prostate normal and cancer cells is shown in Figure 1, MS#1. We found that, of the 6 FABP examined, the fell into 2 groups in terms of the action on prostate cancer cells. Those FABP that were associated with normal prostate cells (PrEC) were A-, H-, and E-FABPs while those associated with cancer prostate cells were I-, L- and sometimes B-FABP. B-FABP and CRABPI were expressed only in a well-differentiated prostate cancer cell line, LNCaP and not in the poorly differentiated cell lines and A-FABP was found to completely disappear in some poorly differentiated prostate cancer cells (DU 145); these three FABPs may provide indicators of stage of prostate cancer. These findings were confirmed in prostate tissues from 15 biopsy samples (Figure 2, MS#1) which were studied in blinded experiments. Additionally, we found that L-FABP could be detected by western blot analysis in the culture fluid from prostate cancer cells but not in the fluid from normal prostate cells (Figure 3, MS#1).

Conclusions from experiments addressing Task 1: We have now shown that the pattern of FABP expression is indicative of whether prostate cells/tissues are normal or cancerous. Furthermore, specific levels of A-, B-FABP and CRABPI may indicate the stage of prostate cancer. We found that L-FABP was secreted into the culture fluid of prostate cancer cells but was not detected in the culture fluid of prostate normal cells. This keeps alive our aim that we may be able to detect the pattern of FABP expression in body fluids (urine, blood or semen) as a screening method to supplement the PSA test as indicators of stage or degree of aggressiveness of prostate cancer.

c) Statement of Work Task 2 correlated with accomplishments

The objective of this task is to determine the relationship of FABP with cellular regulatory processes to see if FABP are important to prostate cancer. The studies in Task #1 have shown that 3 FABP (E-, H-, A-) are associated with normal prostate cells/tissues while another 3 (I-, L-, B-) are associated with prostate tumor cells/tissues and appear in extracellular fluids of tumor, but not normal prostate cells. Therefore, we need to know the regulatory effects of these two classes of FABP in prostate tumor cells. Please note that this information is being written as a manuscript and is expected to be submitted to a journal for publication within 2 weeks.

Methods:

Transfection of DU145 prostate cancer cells with A-FABP using the Ecdysone inducible system. In order to overexpress A-FABP in prostate cancer cells we used the Ecdysone-Inducible Mammalian Expression System (Invitrogen Corp., Carlsbad, CA). This system is based on a unique regulatory mechanism from the insect *Drosophila* and is illustrated in the diagram in Figure 4, shown below. This regulatory system utilizes a two subunit receptor, the ecdysone receptor (pIND/V5-His-Topo or VgEcR) and the retinoid X receptor (RXR) from mammals. DU145- prostate cancer cells that do not express A-FABP had been transfected with A-FABP cDNA contained in the plasmid vector pIND/V5-His-Topo (Invitrogen Corp.,Carlsbad, CA). A-FABP cDNA was obtained by amplification of A-FABP cDNA from 267B normal prostate neonatal cells using specific designed primers. The ligation of the specific cDNA to the pIND/V5-His-Topo was performed as recommended by the manufacturers. Briefly, the amplified cDNA product containing 3' A overhangs is cloned into the linearized pIND vector by the ligation activity of topoisomerase I within 5 minutes at room temperature.

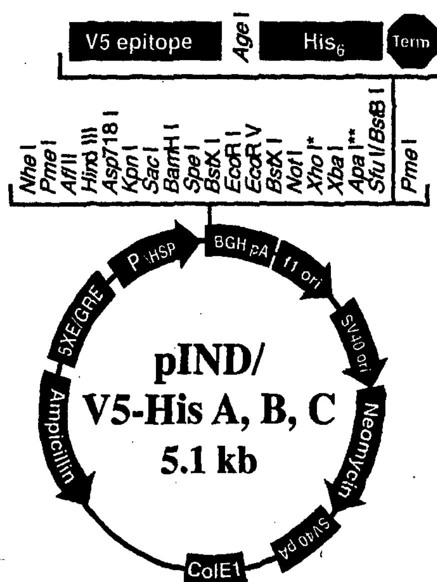


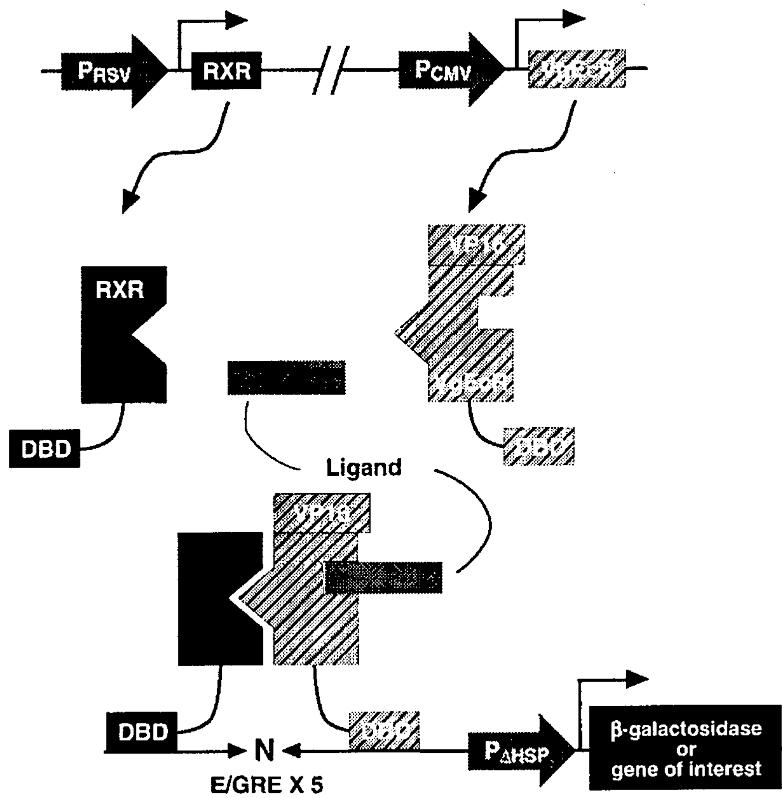
Figure 4. A diagram of the vector system used for transfection of DU145 prostate cancer cells. (Note: in order to avoid confusion, the Figures in the manuscript in the Appendix are referred to as Figures 1-3. Therefore, I am continuing the numbering of Figures starting here with Figure 4 in order not to duplicate figure numbers and inadvertently direct attention to an unintended figure).

DU145 cells were grown in EMEM medium supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine and 1 mM sodium pyruvate. Transfection of DU145 cells with pIND/V5-His-Topo containing a-FABP and the RXR subunit was done using transfectene, a non-liposomal lipid from Qiagen Inc., Valencia, CA. Transfected DU145 cells were maintained in selective medium containing both, G418 (200 μg/ml) and Zeocin (100 μg/ml) antibiotics.

The ecdysone receptor (pIND/V5-His-Topo) is derived from the natural ecdysone receptor from *Drosophila* and modified to contain the VP16 transactivation domain, and the entire system is diagramed in Figure 5 below. The P-box region of the VgEcR DNA binding domain (DBD) was further modified to recognize one half-site from the glucocorticoid response element (5' -AGAACA-3'). RXR is the mammalian homologue of USP (ultraspiracle) the natural partner to the *Drosophila* ecdysone receptor. This receptor recognizes one half-site from the natural ecdysone reposne element (5'-AGTGCA-3'). Both of these receptors are expressed from the pVgRXR vector. The RXR receptor binds the ecdysone response element (VgEcR) in the presence of an inducing molecule, such as ecdysone or an ecdysone analog (ponasterone A was used at 5, 10 and 20 μM). Transcription from a minimal heat shock promoter is activated and this drives the expression of a heterologous gene (a-FABP). Basal levels of transcription in this system are very low since mammalian cells are not responsive to the inducing agent and do not contain the ecdysone receptor.

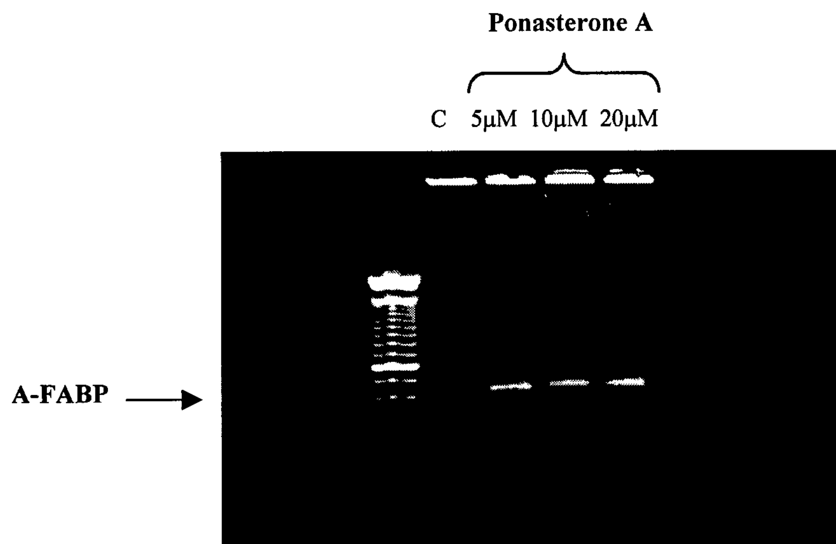
Figure 5. Diagram showing the mechanism by which the expression of the gene A-FABP, when transfected into cells, can be induced.

receptor.



Results, Task 2.

Figure 6. Expression of A-FABP in transfected DU145 prostate cancer cells after exposure to Ponasterone A



The induction of the expression of A-FABP in DU145-clone 4 cells treated with Ponasterone A for 24h was 3-15 fold higher than the non-treated control cells. Also, no expression of A-FABP was observed in the RXR transfected- DU145 cells (Figure 6).

In order to study the possible role of A-FABP in DU145 prostate cancer cell growth, cell proliferation was assessed in non-confluent transfected DU 145 cell cultures using WST-1 reagent (Boehringer-Mannheim, Indianapolis,IN). Briefly, DU145 cells were plated in 96-well plates at 2 x 10^3 cells per well in 1% FBS containing medium. The cells were incubated with 5 µM Ponasterone A for 72 h. WST-1 was added during the last 4 h of the incubation period as recommended by the manufacturers. The color developed was quantified using an ELISA reader and read at 450 nm

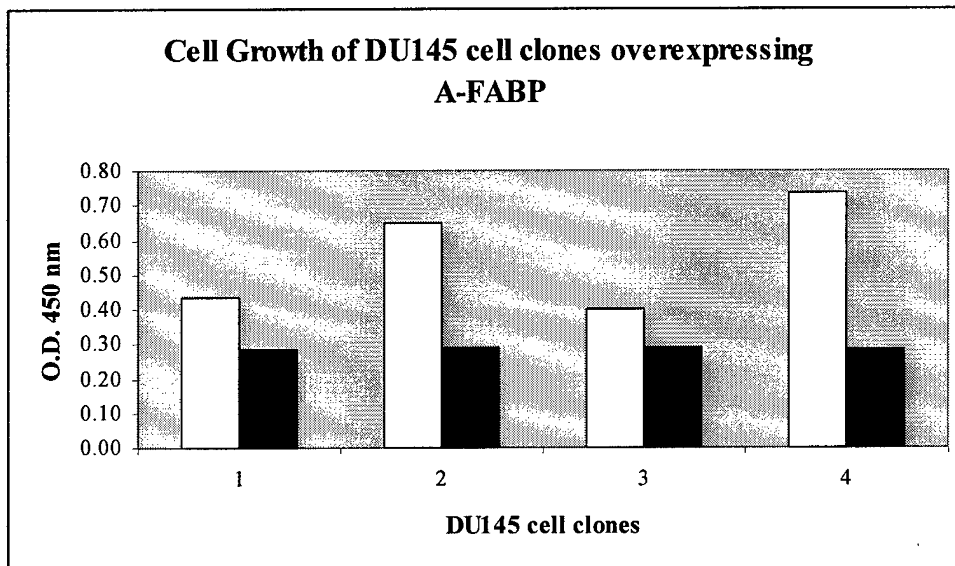


Figure 7. Cell growth analysis of 4 different clones of DU145 cells transfected with A-FABP in an inducible vector. Cell growth inhibition was observed when transfected DU145 cell clones were exposed to 5µM Ponasterone A (■) when compared to cells that were not treated with the hormone (□). Clone number 4 showed a 2.6-fold decrease in cell proliferation when exposed to Ponasterone A. This clone showed a 15-fold A-FABP gene expression increase, the highest of all the clones analyzed.

Detection of apoptotic cells in transfected DU145 cell cultures

In order to find out if the cell growth inhibition effect observed in DU145 cells overexpressing A-FABP was due to a cell death phenomenon, the rate of apoptosis was determined in DU145 prostate cancer cell cultures. Morphological changes in the nuclear condensation of chromatin of cells undergoing apoptosis were assessed by staining with the DNA-binding fluorochrome bis-benzimide (Hoechst 33258; Sigma) as described elsewhere. In brief, transfected DU145 cells were plated in polysterene chamber slides (LabTek) at 5 x 10^4. Transfected DU145 cells from clone number 4 were used. The cells were then kept for 48 h in medium and then treated with or without Ponasterone A at different concentrations in the presence of 10% FBS for different times. After treatment, the cells were washed twice with PBS and incubated with 3.5% paraformaldehyde in PBS for 20 min at room temperature. After fixation, the cells were washed twice with PBS and stained with *bis*-benzimide (16 µg/ml) in PBS. Following 15 min of incubation at room temperature, the cells were washed twice with PBS and the average number of nuclei per field was scored for the incidence of apoptotic chromatin under a fluorescence microscope. Cells with three or more condensed chromatin fragments were considered apoptotic.

Transfected DU145 cells were exposed to Ponasterone A at 5, 10 and 20 μM for 96h. Apoptotic features were observed with concentrations as low as 5 μM. White arrows point at nuclei from cells undergoing apoptosis. As a control, cultures from DU145 cell clone number 2 were also found to undergo apoptosis when A-FABP expression was induced (photomicrograph is shown in Figure 8, MS in preparation #2. Appendix).

The timecourse of the apoptotic effect induced by overexpression of A-FABP in DU145 prostate cancer cells was also assessed by *bis*-benzimide nuclei staining. Three random fields per exposure were analyzed. The number of experiments performed independently were two per each condition.
Apoptosis was found after 48h of exposure to Ponasterone A in transfected DU145 cell cultures. The apoptotic index increased with the amount of Ponasterone A and the time of exposure (Table 2, below).

TABLE 2.
Quantification of Apoptosis in DU145 prostate cancer cells overexpressing A-FABP

	% Apoptotic Cells		
Ponasterone A	***48h***	***72h***	***96h***
0 μM	*3*	*4*	*5*
5 μM	*5*	*13*	*13*
10 μM	*7*	*12*	*19*
20 μM	*8*	*25*	*28*

To find out the possible mechanism by which A-FABP is able to induce apoptosis in DU145 prostate cancer cells, the gene expression of epidermal growth factor (EGF) and transforming growth factor-a (TGF-α) was assessed. These two growth factors are able to recognize and activate the EGF receptor, and therefore, elicit a survival response cells. It has been shown that TGF-α is an autocrine factor in DU145 cells, essentially involved in cell growth. Instead, DU145 cells do not proliferate when exposed to EGF. In DU145 cells overexpressing A-FABP, the levels of TGF-α are reduced more than two-fold, suggesting a role for A-FABP in interfering with the autocrine production of TGF-α and as a consequence, less survival factors are available for DU145 cancer cells. The upregulation of the levels of EGF in DU145 cells overexpressingA-FABP may be a response to the lack of EGF receptor signaling in these cells.

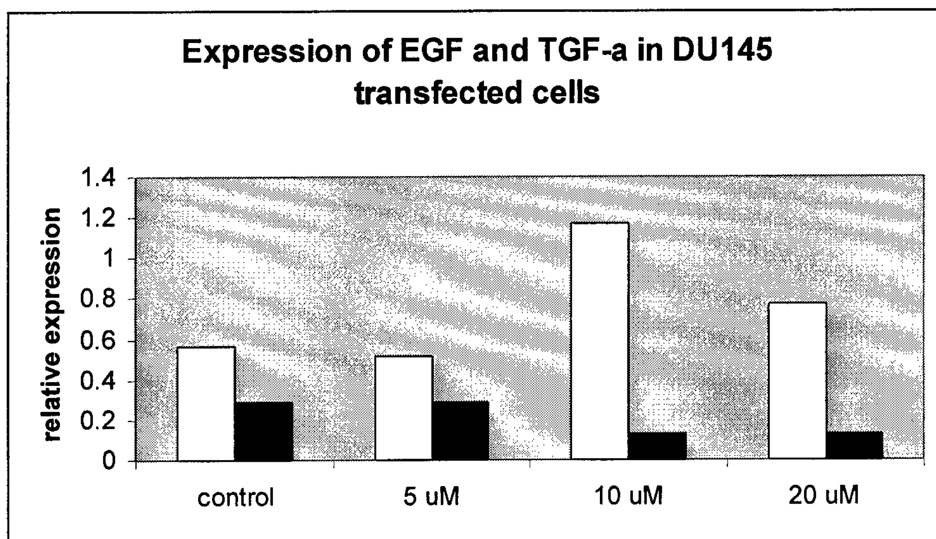


Figure 9. Gene expression of TGF-α (■) and EGF (□) was determined in transfected DU145 prostate cancer cells exposed to Ponasterone A. RT-PCR methodology was used to assess the levels of gene expression.

Further experiments are being planned to elucidate the possible pathway/s involved in the apoptotic effect of A-FABP in prostate cancer cells.

Conclusions from experiments addressing Task 2. The main objective of this task is to verify the importance of FABPs in prostate cancer and to determine their regulatory function and how they are regulated. At this point in time, we have answered the first of those two questions fully, but work is in progress to gain information regarding regulation of FABP by hormones, growth factors and bioactive lipids.

The study was designed to ask the question, "If we increase the expression of A-FABP in prostate cancer cells where the level of A-FABP are low or absent, will the cells become non-cancer like?". We found that to be the case. In these very elegant experiments, A-FABP was transfected into prostate cancer cells using an inducible system so that A-FABP is expressed only when the inducer, ponasterone is present. When A-FABP expression is turned on, the cancer cells stop rapid proliferation and become apoptotic. In addition to this study, we have preliminary data to show that antisense blocked expression of L-FABP and also induced apoptosis in prostate cancer cells. It is important to remember that prostate normal cells already express high levels of A-FABP and very low levels of L-FABP. So these experiments were designed to make the prostate cancer cells resemble normal cells in regards to either A- or L-FABP. Indeed, using either approach caused the cancer cells to cease rapid proliferation and we have preliminary indications that these cells may have lost tumorigenicity (that aspect will not be able to be investigated further due to funding and time limitations). Furthermore, we observed the inter-relationships with growth regulatory systems such as that seen with the EGF receptor and TGF-α. We are currently setting up experiments to examine the reverse aspect, the effect of growth regulators, hormones etc on FABP expression (also outlined in Task 2). These experiments indicate that FABPs and their regulatory pathways are intimately involved in and may serve as therapeutic targets for prostate cancer.

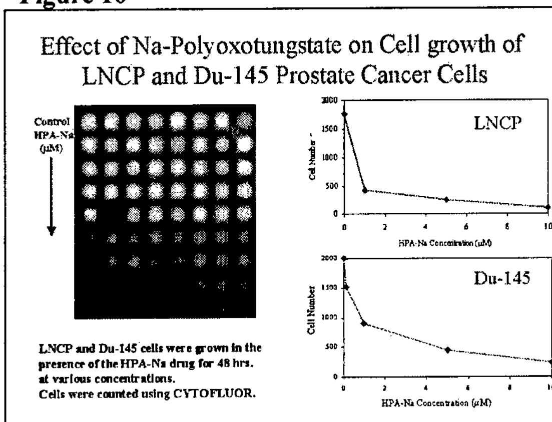
d) Statement of Work Task 3 correlated with accomplishments

Methods: Heteropolyanions (HPA) have been synthesized in our lab and studied as free-radical scavengers that have antiproliferative effects on tumor cells. Cell proliferation was determined by the Cytolite methods (Molecular Probes, San Diego, CA) in which nuclear DNA of surviving cells are quantitated by

staining with propidium iodide. After brief treatment with HPA-Na, and prior to the cells exhibiting damage, we determined the expression levels of various FABP by the methods described in MS#1, Appendix. We are currently setting up the experiment to test HPA effectiveness in the SCID mouse xenograft model using PC-3 prostate cancer cells.

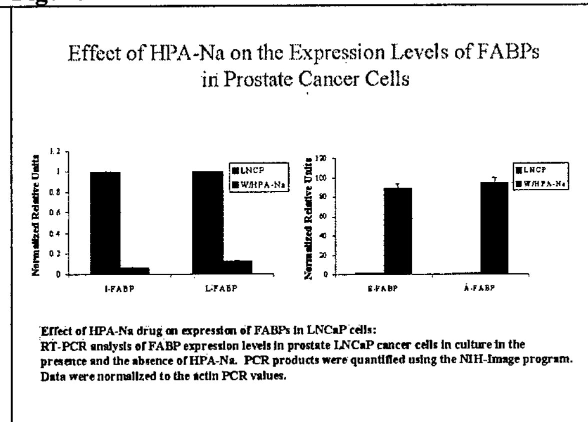
Results:
Figure 10 shows that Na-polyoxotungstate (heteropolyanion, HPA) blocked proliferation of two different prostate cancer cell lines at levels of 1-2 ug/ml. This range has been shown by us previously to be 10-15 fold under toxic levels in bone marrow colony formation assays. After incubation of the cells with HPA-Na for 24 h, FABP expression levels were analyzed. Not surprisingly, expression levels of L- and I-FABP were decreased upon this exposure to the HPA (Figure 11). The interesting aspect was that expression levels of A- and E-FABP dramatically increased relative to those seen prior to exposure to HPA-Na (Figure 11).

Figure 10



LNCP and Du-145 cells were grown in the presence of the HPA-Na drug for 48 hrs. at various concentrations. Cells were counted using CYTOFLUOR.

Figure 11



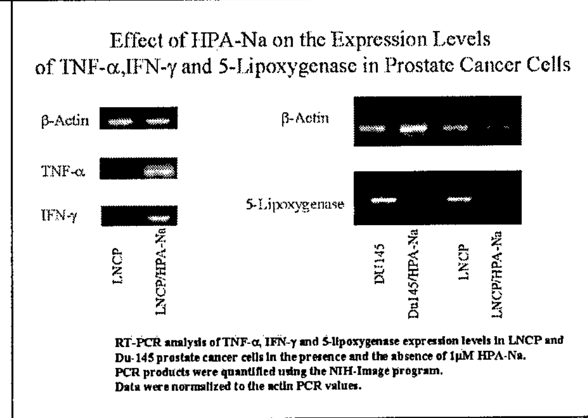
Effect of HPA-Na drug on expression of FABPs in LNCaP cells:
RT-PCR analysis of FABP expression levels in prostate LNCaP cancer cells in culture in the presence and the absence of HPA-Na. PCR products were quantified using the NIH-Image program. Data were normalized to the actin PCR values.

Figure 12

Genes Identified with DD-PCR for LNCP Prostate Cancer Cells Treated with the HPA-Na Drug

Gene	Alternative Titles	Properties	Modulation
1	RNA polymerase II, Transcription Factor	32-kD protein; required for activated transcription; coactivator of the p53 protein	↓ Regulation
2	Cytochrome dependent enzyme	57-kD protein; component of a Complex of the Electron Transport Chain (ETS); involved in oxidative phosphorylation	↓ Regulation
3	Rho Protein	Regulating the dynamics of the actin cytoskeleton and its reoganization in response to extracellular stimuli	↑ Regulation
4	Necrosis Factor		↑ Regulation
5	Ribosomal Protein	Involved in the protein translation machinery	↓ Regulation
6	5-LO	Involved in the metabolism of Arachidonic acid	↓ Regulation

Figure 13



RT-PCR analysis of TNF-α, IFN-γ and 5-lipoxygenase expression levels in LNCP and Du-145 prostate cancer cells in the presence and the absence of 1μM HPA-Na. PCR products were quantified using the NIH-Image program. Data were normalized to the actin PCR values.

Figures 12 and 13 show results of studies of global gene expression in response to HPA-Na treatment of prostate cancer cells. In general, Figure 12 indicates that apoptosis genes are activated in response to HPA-Na exposure and Figure 13 shows demonstrable changes in mediators in response to HPA-Na.

Conclusions: HPA-Na is a very promising drug for possible use in treatment of prostate cancer and the mechanism of its regulation of proliferation appears to center on induction of apoptosis in the prostate cancer cells. The key test is in progress. We are using SCID mice carrying a xenograft of human PC-3 cells. We will attempt to treat the mice to determine if the HPA-Na effectively stops tumor growth/development at concentrations non-toxic to the mice.

e) SOW

With notations (italicized and bold) showing what tasks have been completed, which have not been successful and which studies are in progress.

Task 1. Examine the type(s) and levels of fatty acid binding proteins (FABPs) to determine if there is a correlation between normal and the various stages of prostate cancer and the FABPs (1-18 months)

Quantitative description of various FABPs in different stages of prostate cancer. Our preliminary evidence obtained using 3 RT-PCR primers pairs suggests that there is a major difference in quantity of FABPs and may be a difference in the type of FABPs between normal and tumor prostate cells. It is important to clarify and extend these findings.

- Continue to design specific PCR primers for the different class of FABPs and use them in cultured cells from various stages of prostate cancer. Characterize the probes for there selectivity/cross-reactivity with the different families of FABP's. Select a battery of primers which will be used for further screening. (1-6 months) ***Objective completed as described***
- Using these selected primers, adapt to the methodology for quantitative PCR using non-radioactive probes such as the attomolar sensitive aqua-lite system currently in use in our laboratories. (3-12 months). ***This approach was unsuccessful and was abandoned for ELISA (next obj).***
- Use currently available antisera to L-FABP, and to other FABP's as available. Identify the type and levels of these protein(s) in normal and tumor cell cultures and conditioned medium using Western blots, and, if appropriate, develop an ELISA assay for measurement of quantities of FABPs (2-10 months). ***Currently available antisera do not discriminate between the FABPs because they share similarities in 3-dimensional structure. Some FABPs share a great deal of amino acid homology. The only one antisera that so far seems to be specific is one we have to L-FABP.***
- Antisera against prostate-specific FABP(s) will be raised if it is determined to be necessary (months 10-16***). Standard methods to produce discriminating antisera (specific to each FABP) have failed. It is necessary to employ new unconventional approachs that we have designed to take advantage of certain structural features of each FABP. We will not have sufficient time or funds to investigate this further under the auspices of this grant.***

Characterization of functional activities of FABP's in normal vs tumor prostate cell cultures. Binding of arachidonic acid to FABP's has been shown to increase dramatically in tumor vs normal cells in hepatic cancer.

- Measure the levels of various eicosanoids and AA bound to these FABPs from prostate tumor and normal cell lines (months 11-18). ***This approach has not shown significant differences between FABPs but probably requires more sophisticated approaches than we can employ with the funds available***
- Analyze the role of PPAR and RXR with prostate FABP in cultured cells by using reagents (from our previous studies of L-FABP) which target these receptors (months 15-18). ***We are in the process of setting up experiments using reagents that target PPAR to observe the effects on FABPs***

Localization of FABP in prostate normal vs tumor cells from tissue culture and from biopsy samples in an effort to determine if the pattern of its expression differs in normal vs different stages of prostate cancer.

- Establish a method for In situ PCR detection of FABP levels in prostate normal and various stages of tumor cultured cells. Optimize the conditions and characterize subcellular compartmentalization of these FABP's. (6-15 months) ***We have carried out this study using antisense to fluorescently tagged L- or B-FABP and those data are being assembled, but are not presented here.***
- Perform In situ PCR on biopsy samples of prostate normal vs tumor (16-30 months). ***We have not done this yet***

Task 2. Importance of FABP to prostate cancer. To examine the effect of exogenous addition of fatty acids, hormones and growth factors and other AA metabolites on growth, signaling and levels of FABPs in cultured prostate cells (months 19-30***). We realized that we could gain much of this information by asking the question in a more straight-forward manner. If A-FABP levels are increased in tumor cells, does it make them normal-like? If L-FABP expression is blocked (as with antisense to the message), do the tumor cells become normal-like?***

Part 1 of new question: A-FABP has been transfected into DU145 prostate cancer cells and found to regulate cell growth and cause the cells to become normal-like. Part 2. We have carried out the antisense experiments and the data will be presented in the next report

- Perform growth assays in cells in the presence of various agents involved in the regulation of FABPs (19-21 months). ***These experiments are being planned.***
- Using the conditions established above, measure the levels of FABPs in response to growth factors, eicosanoids and hormones that are known to modulate the growth of these cancer cells (22-30 months)
- Assay the activity of various signaling kinases such as raf, MAPK, JNK, etc. (19-30 months)
- Measure the levels of various eicosanoids by HPLC methods and measure the levels of 5-lipoxygenase and FLAP in an attempt to find a correlation between the AA pathway and the FABPs for specific stages of prostate cancer first in tumor cell lines characteristic of various stages of cancer progression and then in biopsy samples (22-30 months). ***Some of this has been done and will be written up.***

Task 3. To study the effect of heteropolyanion drugs (developed in our laboratory) alone or in combination with inhibitors of eicosanoid metabolism on the growth of prostate cancer cells (months 9-30)

- Perform growth assays in the presence of various concentrations of heteropolyanion drugs alone or in combination with various inhibitors of eicosanoid metabolism (9-12 months). ***Completed***
- Assay the levels of FABPs in response to these drugs (13-18 months). ***Completed***
- Measure the levels of various eicosanoids in response to the heteropolyanion drugs (15-30) ***Completed***
- Examine the PPAR and RXR interaction with the FABPs using gel shift analysis for PPRE before and after the drug treatment (24-30 months) . ***Some of this has been done***
- Use a SCID mouse model for prostate cancer (using xenographs of LNCAP cells) and study the therapeutic effects of these various drug(s) alone or in combinations (18-30 months). ***We are in the process of carrying out this study***

(7) Key Research Accomplishments: a) Established that the pattern of FABP expression is different for prostate cancer vs normal cells and that pattern was reflected in prostate biopsy sample tissues from patients. b) Determined that prostate cancer cells secreted L-FABP into their culture fluid while normal cells did not. c) Successfully transfected an inducible construct containing the gene for A-FABP and those studies demonstrated that FABP regulation was critical for prostate cancer cells to survive. d) Found that a series of drugs (HPAs) synthesized by us effectively blocked proliferation of prostate cancer cells at concentrations 10-15-fold lower than toxic levels. These findings are being confirmed in SCID mice bearing xenografts of human prostate cancer cells.

(8) Reportable Outcomes

a) Bibliography: Manuscripts, abstracts, patents

Manuscript s

1. Das, Hammamieh, Melhem and Jett. Distinct Fatty Acid Binding Protein Profile In Prostate Normal and Cancer Cells and Tissues. **MS #1 in Appendix (submitted to Cancer Research)**
2. De Santis, Das and Jett. Adipocyte Fatty Acid Binding Protein Induces Apoptosis upon Induction of Expression in Transfected DU 145 Prostate Cancer Cells. MS in text under TASK 2, in preparation for submission within a few weeks.

Abstracts:

1. Rasha Hammamieh, Rina Das, Mona Melhem and Marti Jett (1999). Distinct Fatty Acid Binding Protein Profile In Prostate Normal And Tumor Tissues. FASEB J. 13:A1462 (#751).
2. Rasha Hammamieh, Rina Das and Marti Jett.(1999). Alteration of FABP patterns by heteropolyanions in prostate cancer. Proceedings of the American Association for Cancer Research 40:2(#10)
3. Rina Das, Apsara Dhokalia and Marti Jett (1999). Expression pattern of different fatty acid binding proteins in cancer cells. Proceedings of the American Association for Cancer Research 40:363(#2405)

Patent Disclosure submitted Nov 1998; Patent filed Nov 1999:

M. Jett, R. Das, R. Neill. Fatty acid binding proteins as diagnostic markers for breast and prostate cancer. Pending

b) **Development of cell lines:** A novel cell reagent has been developed, the A-FABP inducible construct transfected into prostate cancer cell line, DU145

c) **Animal models used:** SCID mice with a PC3 prostate cancer cell xenograft

d) **Personnel receiving pay from this grant:**
Professional Staff: Dr. Marta DeSantis, Dr. Rina Das, Dr. Rasha Hammamieh
Student interns: Srinivas Sidhara, Julia Gowan, Rahul Dasgupta, Tong Vi Li, Roy Hwang, Michael Hartl

(9) Conclusions: a) We have shown that there is a distinct pattern of FABP expression in prostate cancer vs normal cells and that finding has be confirmed in patient samples of biopsy specimens. b) Certain FABP may be markers that can be exploited to reveal stage or aggressiveness of prostate cancer. c) Since FABP are secreted from cells, we have shown that liver FABP was found in the extracellular fluid of prostate cancer but not prostate normal cells and this characteristic may provide an opportunity to use FABP patterns in body fluids as markers for prostate cancer and stage specific screening. d) Transfection of A-FABP into prostate cancer cells (that lack A-, but have abundance of L-, and I-FABP) caused the cells to become more normal-like; they showed decreased proliferation and induction of genes involved in apoptosis. Similar results were seen upon using antisense to L-FABP to accomplish the same end results. Those findings indicate that FABP are important in prostate cancer cell regulation. e) Heteropolyanions (HPA) show good potential for therapeutic intervention in prostate cancer and key experiments are underway to verify those results *in vivo.*

(10) References

1. Astron A, Tavakkol A, Pettersson U, Cromie M, Elder JT and Voorhees JJ.: Molecular cloning of two human cellular retinoic acid-binding proteins (CRABP). Retinoic acid-induced expression of CRABP-II but not CRABP-I in adult human skin in vivo and in skin. J Biol Chem 266:17662-17666, 1991.
2. Avis I, Jett M, Boyle T, Vos MD, Moody T, Treston A, Martinez A and Mulshine J.: Growth control of lung cancer by interruption of 5-lipoxygenase-mediated growth factor signaling. J Clin Invest 97:806-813, 1996.
3. Avis I, Jett M, Vos M and Mulshine J.: Growth control of breast cancer by interruption of the lipoxygenase pathway of arachidonic metabolism. Abstr Proceed Am Assn Cancer Res 37:4115, 1996.
4. Banaszak L, Winter N, Xu Z, Bernlohr DA, Cowan S and Jones TA.: Lipid-binding proteins: a family of fatty acid and retinoid transport proteins. Adv Protein Chem 45:89-151, 1994.
5. Bass NM.: The cellular fatty acid bindng proteins: aspects of structure, regulation and function. Int Rev Cytol 111:1988.
6. Baxa CA, Sha R, S., Buelt MK, Smith AJ, Matarese V, Chinander LL, Boundy kL and Bernlohr DA.: Human adipocyte lipid-binding protein: purification of the protein and cloning of its complementary DNA. Biochem 28:8683-8690, 1989.
7. Bernier M, Laird DM and Lane MD.: Insulin-activated tyrosine phosphorylation of a 15-kilodalton protein in intact 3T3-L1 adipocytes. Proc Natl Acad Sci U S A 84:1844-8, 1987.
8. Boyle T, Lancaster V, Hunt R, Gemski P and Jett M.: Method for simultaneous isolation and quantitation of platelet activating factor and multiple arachidonate metabolites from small samples: analysis of effects of Staphylococcus aureus enterotoxin B in mice. Anal Biochem 216:373-382, 1994.
9. Boyle T, Welch C, McMorrow S, Gemski P and Jett M.: Arachidonate metabolism in staphylococcal entertoxin B-stimulated cultures of human monocytes and lymphocytes: specific inhibitors repress biological responses. Abstr Ann Meet Am Soc Microbiol 95:B-45, 1995.
10. Buelt MK, Shekels LL, Jarvis BW and Bernlohr DA.: In vitro phosphorylation of the adipocyte lipid-binding protein (p15) by the insulin receptor. Effects of fatty acid on receptor kinase and substrate phosphorylation. J Biol Chem 266:12266-71, 1991.

11. Celis JE, Ostergaard M, Basse B, Celis A, Lauridsen JB, Ratz GP, Andersen I, Hein B, Wolf H, Orntoft TF and Rasmussen HH.: Loss of adipocyte-type fatty acid binding protein and other protein biomarkers is associated with progression of human bladder transitional cell carcinomas. Cancer Res 56:4782-90, 1996.
12. Chaudry A, McClinton S, Moffat LE and Wahle KW.: Essential fatty acid distribution in the plasma and tissue phospholipids of patients with benign and malignant prostatic disease. Br J Cancer 64:1157-60, 1991.
13. Chaudry AA and Dutta-Roy AK.: Purification and characterization of a fatty acid binding protein from human prostatic tissue. Lipids 28:383-8, 1993.
14. Chaudry AA, Wahle EWJ, McClinton S and Moffat LWF.: Arachidonic acid metabolism in benign and malignant prostatic tissue in vitro: effects of fatty acids and cyclooxygenase inhibitors. Int J Cancer 57:176-180, 1994.
15. Colantuoni V, Cortese R, Minsson M, Lundvall J, Bavik CO, Eriksson U, Peterson PA and Sundelin J.: Cloning and sequencing of a full length cDNA corresponding to human cellular retinol-binding protein. Biochem Biophys Res Comm 130:431-439, 1985.
16. Custer RP and Sorof S.: Target polypeptide of a carcinogen is associated with normal mitosis and carcinogen-induced hyperplasias in adult hepatocytes. Proc Natl Acad Sci USA 81:7638-6742, 1984.
17. Custer RP and Sorof S.: Mitosis in hepatocytes is generally associated with elevated levels of the target polypeptide of a liver carcinogen. Differentiation 30:176-181, 1985.
18. Das R and Vonderhaar BK.: Transduction of prolactin's growth signal through both long and short forms of the prolactin receptor. Mol Endocrinol 9:1750-1759, 1995.
19. Das R and Vonderhaar BK.: Involvement of SHC, Grb2 and Sos in prolactin signal transduction. Oncogene 13:1139-1145, 1996.
20. Davidson NO, Ifkovits CA, Skarosi SF, Hausman AM, Llor X, Sitrin MD, Montag A and Brasitus TA.: Tissue and cell-specific patterns of expression of rat liver and intestinal fatty acid binding protein during development and in experimental colonic and small intestinal adenocarcinomas. Lab Invest 68:663-75, 1993.
21. Eller MS, Oleksiak MF, McQuaid TJ, McAfee SG and Gilchrest BA.: The molecular cloning and expression of two CRABP cDNAs from human skin. Exp Cell Res 198:328-336, 1992.
22. Franceschi S.: Fat and prostate cancer. Epidemiol 5:271-273, 1994.
23. Ghosh J and Myers EE.: Arachidonic acid stimulates prostate cancer cell growth: critical role of 5-lipoxygenase. Biochem Biophys Res Comm 235:418-423, 1997.
24. Giovannucci E, Rimm EB, Colditz GA, Stampfer MJ, Ascherio A, Chute CC and Willett WC.: A prospective study of dietary fat and risk of prostate cancer. J Nat Cancer Inst 85:1571-1579, 1993.
25. Gorry M, Gabbaizedh D, Furey W, Gates L, Preston R, Aston C, Yingze Z, Ulrich C, Ehrlich G and Whitcomb D.: Mutations in the cationic trypsinogen gene are associated with recurrent acute and chronic pancreatitis. Gastroenterology 113:1063-1068, 1997.
26. Grosse R, Boehmer FD, Langen P, Kurtz A, Lehmann W, Mieth M and Wallukat G.: Purification, biological assay and immunoassay of mammary-derived growth inhibitor. Methods Enzymol 198:425-440, 1991.
27. Grosse R, Bohmer F-D, Binas B, Kurtz A, Spitzer E, Muller T and Zschiesche W.: Mammary derived growth inhibitor. In R. B. Dickson and M. Lippman, eds Cancer Treatment and Research . Genes Oncogenes and Hormones. Boston, 1992, pp 69-97.
28. Grosse R and Langen P.: Mammary derived growth inhibitor. In M. Sporn and A. Roberts, eds Handbook of Experimental Pharmacology. Heidelberg, 1990, pp 249-265.
29. Harvei S, Bjerve KS, Tretli S, Jellum E, Robsahm TE and Vatten L.: Prediagnostic level of fatty acids in serum phospholipids: Ω-3 and Ω-6 fatty acids and the risk of prostate cancer. Int J Cancer 71:545-551, 1997.
30. Hayasaka K, Nanao K, Tahara M, Sato W, Takada G, Miura M and Uyemura K.: Isolation and sequence determination of cDNA encoding P2 protein of human peripheral myelin. Biochem Biophys Res Comm 181:204-207, 1991.
31. Hresko RC, Bernier M, Hoffman RD, Flores-Riveros JR, Liao K, Laird DM and Lane MD.: Identification of phosphorylated 422(aP2) protein as pp15, the 15-kilodalton target of the insulin receptor tyrosine kinase in 3T3-L1 adipocytes. Proc Natl Acad Sci U S A 85:8835-9, 1988.

32. Hresko RC, Hoffman RD, Flores-Riveros JR and Lane MD.: Insulin receptor tyrosine kinase-catalyzed phosphorylation of 422(aP2) protein. Substrate activation by long-chain fatty acid. J Biol Chem 265:21075-85, 1990.
33. Huynh H, Alpert L and Pollak M.: Silencing of the mammary-derived growth inhibitor (MDGI) gene in breast neoplasms is associated with epigenetic changes. Cancer Res 56:4865-70, 1996.
34. Jett M, Brinkley W, Neill R, Gemski P and Hunt R.: Staphylococcus aureus enterotoxin B challenge of monkeys: correlation of plasma levels of arachidonic acid cascade products with occurrence of illness. Infect Immun 58:3494-3499, 1990.
35. Kaikaus RM, Bass NM and Ockner RK.: Functions of fatty acid binding proteins. Experientia 46:617-630, 1990.
36. Keler T, Barker CS and Sorof S.: Specific growth stimulation by linoleic acid in hepatoma cell lines transfected with the target protein of a liver carcinogen. Proc Natl Acad Sci U S A 89:4830-4, 1992.
37. Keler T, Khan SH and Sorof S.: Liver fatty acid binding protein and mitogenesis in transfected hepatoma cells. In K. V. Honn, L. J. Marnett and S. Nigam, eds Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation and Radiation Injury. Boston, 1995, pp 517-524.
38. Keler T and Sorof S.: Growth promotion of transfected hepatoma cells by liver fatty acid binding protein. J Cell Physiol 157:33-40, 1993.
39. Khan SH and Sorof S.: Liver fatty acid-binding protein: specific mediator of the mitogenesis induced by two classes of carcinogenic peroxisome proliferators. Proc Natl Acad Sci U S A 91:848-52, 1994.
40. Kurtz A, Vogel F, Funa K, Heldin C-H and Grosse R.: Developmental regulation of mammary-derived growth inhibitor expression in bovine mammary tissue. J Cell Biol 110:1779-1789, 1990.
41. Loughney AD, Kumarendran MK, Thomas EJ and Redfern CPF.: Variation in the expression of cellular retinoid binding proteins in human endometrium throughout the menstrual cycle. Hum Reprod 10:1297-1304, 1995.
42. Lowe JB, Boguski MS, Sweetser DA, Elshourbagy NA, Taylor JM and Gordon JI.: Human liver fatty acid binding protein. Isolation of a full length cDNA and comparative sequence analyses of orthologous and paralogous proteins. J Biol Chem 260:3413-7, 1985.
43. Maatman RG, van Moerkerk HT, Nooren IM, van Zoelen EJ and Veerkamp JH.: Expression of human liver fatty acid-binding protein in Escherichia coli and comparative analysis of its binding characteristics with muscle fatty acid-binding protein. Biochim Biophys Acta 1214:1-10, 1994.
44. Madsen P, Rasmussen HH, Leffers H, Honore B and Celis JE.: Molecular cloning and expression of a novel keratinocyte protein (psoriasis-associated fatty acid-binding protein [PA-FABP]) that is highly up-regulated in psoriatic skin and that shares similarity to fatty acid-binding proteins. J Invest Dermatol 99:299-305, 1992.
45. Oelkers P and Dawson PA.: Cloning and chromosomal localization of the human ileal lipid-binding protein. Biochim Biophys Acta 1257:199-202, 1995.
46. Ostergaard M, Rasmussen HH, Nielsen HV, Vorum H, Orntoft TF, Wolf H and Celis JE.: Proteome profiling of bladder squamous cell carcinomas: identification of markers that define their degree of differentiation. Cancer Res 57:4111-7, 1997.
47. Peeters RA, Ena JM and Veerkamp JH.: Expression in Escherichia coli and characterization of the fatty-acid-binding protein from human muscle. Biochem J 278:361-4, 1991.
48. Peeters RA, Veerkamp JH, Geurts van Kessel A, Kanda T and Ono T.: Cloning of the cDNA encoding human skeletal-muscle fatty-acid-binding protein, its peptide sequence and chromosomal localization. Biochem J 276:203-7, 1991.
49. Prinsen CFM and Veerkamp JH.: Transfection of L6 myoblasts with adipocyte fatty acid-binding protein cDNA does not affect fatty acid uptake but disturbs lipid metabolism and fusion. Biochem J 329:265-273, 1998.
50. Rasmussen HH, Orntoft TF, Wolf H and Celis JE.: Towards a comprehensive database of proteins from the urine of patients with bladder cancer. J Urol 155:2113-9, 1996.
51. Raza H, Pongubala JR and Sorof S.: Specific high affinity binding of lipoxygenase metabolites of arachidonic acid by liver fatty acid binding protein. Biochem Biophys Res Commun 161:448-55, 1989.
52. Rose DP and Connolly JM.: Effects of fatty acids and eiconsanoid synthesis inhibitors on the growth of two human prostate cancer cell lines. Prostate 1811243-254:1991.
53. Sato N, Gleave ME, Bruchovsky N, Rennie PS, Beraldi E and Sullivan LD.: A metastatic and androgen-sensitive human prostate cancer model using intraprostatic inoculation of LNCaP cells in SCID mice. Cancer Res 57:1584-1589, 1997.

54. Serhan CN.: Lipoxin biosynthesis and its impact in inflammatory and vasuclar events. Biochim Biophys Acta 1212:1-25, 1994.
55. Shearman MS, Naor Z, Sekiguchi K, Kishimoto A and Nishizuka Y.: Selective activation of the γ-subspecies of protein kinase C from bovine cerebellum by arachidonic acid and its lipoxygenase metabolites. FEBS Lett 243:177-182, 1989.
56. Shimizu F, Watanabe TK, Shinomiya H, Nakamura Y and Fujiwara T.: Isolation and expression of a cDNA for human brain fatty acid-binding protein (B-FABP). Biochim Biophys Acta 1354:24-8, 1997.
57. Snowdon DA, Phillips RL and Choi W.: Diet obesity and risk of fatal prostatic cancer. Amer J Epidemiol 2:244-250, 1984.
58. Sohmiya K, Tanaka T, Tsuji R, Yoshimoto K, Nakayama Y, Hirota Y, Kawamura K, Matsunaga Y, Nishimura S and Miyazaki H.: Plasma and urinary heart-type cytoplasmic fatty acid-binding protein in coronary occlusion and reperfusion induced myocardial injury model. J Mol Cell Cardiol 25:1413-26, 1993.
59. Sorof S.: Modulation of mitogenesis by liver fatty acid binding protein. Cancer Metastasis Rev 13:317-36, 1994.
60. Specht B, Bartetzko N, Hohoff C, Kuhl H, Franke R, Borchers T and Spener F.: Mammary derived growth inhibitor is not a distinct protein but a mix of heart-type and adipocyte-type fatty acid-binding protein. J Biol Chem 271:19943-9, 1996.
61. Sweetser DA, Birkenmeier EH, Hoppe PC, McKeel DW and Gordon JI.: Mechanisms underlying generation of gradients in gene expression within the intestine: an analysis using transgenic mice containing fatty acid binding protein-human growth hormone fusion genes. Genes Dev 2:1318-32, 1988.
62. Sweetser DA, Birkenmeier EH, Klisak IJ, Zollman S, Sparkes RS, Mohandas T, Lusis AJ and Gordon JI.: The human and rodent intestinal fatty acid binding protein genes. A comparative analysis of their structure, expression, and linkage relationships. J Biol Chem 262:16060-71, 1987.
63. Terry-Koroma B and Vonderhaar BK.: Tamoxifen, prolactin and TGF-beta interactions in ER- breast cancer cell growth. Endocrinology 35:112-115, 1996.
64. Thompson J, Winter N, Terwey D, Bratt J and Banaszak L.: The crystal structure of the liver fatty acid-binding protein. A complex with two bound oleates. J Biol Chem 272:7140-50, 1997.
65. Thumser AE, Voysey J and Wilton DC.: Mutations of recombinant rat liver fatty acid-binding protein at residues 102 and 122 alter its structural integrity and affinity for physiological ligands. Biochem J 314:943-9, 1996.
66. Tipping E and Ketterer B.: The influence of soluble binding proteins on lipophile transport and metabolism in hepatocytes. Biochem J 195:441-52, 1981.
67. Van Nieuwenhoven FA, Kleine AH, Wodzig WH, Hermens WT, Kragten HA, Maessen JG, Punt CD, Van Dieijen MP, Van der Vusse GJ and Glatz JF.: Discrimination between myocardial and skeletal muscle injury by assessment of the plasma ratio of myoglobin over fatty acid-binding protein. Circulation 92:2848-54, 1995.
68. Veerkamp JH, Paulussen RJ, Peeters RA, Maatman RG, van Moerkerk HT and van Kuppevelt TH.: Detection, tissue distribution and (sub)cellular localization of fatty acid-binding protein types. Mol Cell Biochem 98:11-18, 1990.
69. Veerkamp JH, Peeters RA and Matman RGHJ.: Structural and functional features of different types of cytoplasmic fatty acid-binding proteins. Biochim Biophys Acta 1081:1-24, 1991.
70. Vork MM, Glatz JFC and Van Der Vusse GJ.: On the mechanism of long chain fatty acid transport in cardiomyocytes as facilitated by cytoplasmic fatty acid-binding protein. J Theoret Biol 160:207-222, 1993.
71. Wang U, Corr JG, Thaler HT, Tao Y, Fair WR and heston WDW.: Decreased growth of established human prostate LNCaP tumors in nude mice fed a low-fat diet. j Nat Cancer Inst 87:1456-1462, 1995.
72. Wodzig KW, Kragten JA, Hermens WT, Glatz JF and van Dieijen-Visser MP.: Estimation of myocardial infarct size from plasma myoglobin or fatty acid-binding protein. Influence of renal function. Eur J Clin Chem Clin Biochem 35:191-8, 1997.
73. Wynder EL, Laakso K, Sotarauta M and Rose D.: Metabolic epidemiology of prostatic cancer. Prostate 5:47-53, 1984.
74. Xu Z, Bernlohr DA and Banaszak LJ.: Crystal structure of recombinant murine adipocyte lipid-binding protein. Biochemistry 31:3484-92, 1992.
75. Yang Y, Spitzer E, Kenney N, Zschiesche W, Li M, Kromminga A, Muller T, Spener F, Lezius A, Veerkamp JH and et al.: Members of the fatty acid binding protein family are differentiation factors for the mammary gland. J Cell Biol 127:1097-109, 1994.

(11) Appendices

EXPRESSION PATTERN OF FATTY ACID BINDING PROTEINS IN HUMAN NORMAL AND CANCER PROSTATE CELLS AND TISSUES

[1]Rina Das, Rasha Hammamieh, Roger Neill, Mona Melhem* and Marti Jett
Walter Reed Army Inst. of Research, Division of Pathology, Washington D:C. 20307
*Pittsburgh Hospital, University of Pittsburgh, PA.

Running title: FABP Expression Patterns in Prostate Cancer Cells

Key words: Fatty acid binding protein, bioactive lipids, gene expression, prostate cancer, diagnostic marker

Abbreviations: Fatty acid binding proteins [FABP]; arachidonic acid [AA].

[1]Corresponding Author:
Dr. Rina Das
Walter Reed Army Institute of Research
503 Robert Grant Rd
Silver Spring, MD 20910

Abstract:

Fatty acid binding proteins (FABP) are a family of cytoplasmic protein that serve as crucial transporters of bioactive lipids, which, in turn, stimulate proliferation. We have analyzed normal and prostate cancer cells and biopsy samples for the presence of specific FABPs by measuring mRNA expression levels of these FABPs. Our results indicate that prostate cells express a variety of different types of FABPs. We found that as a group Liver-FABP and Intestine-FABP were elevated 5-9 fold in most of the cancer cells compared to the normal primary prostate cells. In contrast, Adipose-FABP, Epidermal-FABP and Heart-FABP were severely down regulated (3-50 fold) in cancer cells compared to the normal cells. These observations were confirmed in human tissue samples obtained from prostate cancer patients. We also report of secretion of the L-FABP protein from the prostate cancer cells into the media. We propose that there is a distinct balance between these two groups of FABP whose up or down regulation in cells may play a role in prostate cancer. Furthermore, the pattern of expression of these FABPs may serve as a marker for the degree of aggressive phenotype of prostate cancer.

INTRODUCTION:

Epidemiological studies on carcinoma of the prostate gland have shown a positive relationship between the consumption of dietary fats and development of prostate cancer (6,15,20). Linoleic acid, a common component of dietary fat, and its metabolic derivative, arachidonic acid, have both been associated with prostate tumors and cell proliferation (7,8). Intracellular transport of such bioactive lipids is a critical component in the process by which these molecules continuously stimulate proliferation through interactions with nuclear receptors. Transport and utilization of lipids are mediated by an important family of cytoplasmic proteins, known as fatty acid binding proteins (FABPs) (1,19). Several studies suggest that FABP increase the solubility of fatty acids in the cell cytoplasm causing a net diffusion of fatty acids from the plasma membrane to the intracellular membrane compartments (17).

The members of this multigene family of FABPs consist of at least seven types whose amino acid sequences have been obtained from protein purified from tissues or from cDNA sequences. The FABPs are approximately 14 kD in size encoding about 115 amino acids (1). The designations for each of the FABPs has been derived from the tissue from which it was originally isolated and includes: 1) adipocyte (A-FABP), 2) heart or muscle (H-FABP), 3) brain (B-FABP), 4) epidermis or psoriasis-associated (E-FABP), 5) liver (L-FABP), 6) intestine (I-FABP), and 7) myelin or P2 (P2-FABP). As a group, human A-FABP, H-FABP, B-FAP, and E-FABP share between 50-65% protein sequence homology and contain a tyrosine, near residue 20, that can be phosphorylated (1, 19). Although they are intracellular proteins, specific FABPs have been detected in elevated levels in plasma or urine of patients suffering myocardial infarction or bladder cancer (14,16,18,). FABPs are known to bind many different groups of

fatty acids and their derivatives, including eicosanoids and other bioactive lipids [reviewed in (19)]. L-FABP exhibits different lipid binding characteristics from that of A-FABP or H-FABP.

Certain FABPs have been reported to have differential effects on cell growth when cDNA clones have been transfected into these cells. The expression of L-FABP is elevated in liver during cell proliferation such as carcinogenesis or regeneration (4). Transfection of L-FABP into hepatoma cells increased their proliferation (11,12). In contrast, mammary-derived growth inhibitor (MDGI, also known as H-FABP) appears only in lactating normal and not tumor mammary cells (9). Transfection of a cDNA clone of MDGI into breast cancer cells or mouse mammary epithelial cells results in loss of tumorigenicity (10). Other FABP types have been implicated in control of cell proliferation or cancer (2, 9)

Such studies led us to propose that cells may express different FABP types depending upon their specific states of cell proliferation. We chose to look at prostate normal and cancer cells as a model system. A "liver-like" FABP was reported from prostate cancer tissue (3) suggesting L-FABP may be expressed in these cancer cells. In this study we have examined the levels of FABPs in several prostate normal and cancer cell lines in order to establish a correlation between presence and levels of FABP with the stage of cancer represented by each cell line. The levels of expression of mRNA for five selected FABPs were analyzed using primers designed for RT-PCR. We have found that individual prostate cell lines can express different types of FABPs and that their expression is altered in prostate cancer compared to normal cells. Two heart-type FABPs (A-FABP, E-FABP) were down regulated in cancer cells whereas the liver types (L-FABP, I-FABP) were upregulated in cancer cells. Brain (B-FABP), structurally related to the group known as the heart type FABP, was elevated only in certain types of prostate cancer cells. Similar differences in FABP expression were found in clinical samples of human prostate

normal and cancer tissue. We have also established that these FABP proteins are secreted into the media in tissue culture from prostate cancer cells and may provide a mechanism of identifying aggressiveness of prostate cancer.

MATERIALS AND METHODS:

Reagents: Tissue culture media, custom oligonucleotide DNA primers, TRIZOL and reverse transcriptase were obtained from Life Technologies (Gaithersburg, MD). PCR Master Mix was obtained from Boehringer Mannheim (Indianapolis, IN). PCR-ELISA kit was obtained from KamTek, Inc. (Rockville, MD).

Cell and tissue samples. Culture conditions and media used for individual cell lines was followed as described in manufacturer's instructions. (ATCC, McLean, VA and Clonetics Corp, San Diego, CA). Primary cultures of normal prostate cells were obtained from Clonetics Corp. The prostate cancer cell lines LNCAP, PC3, DU145 were obtained from ATCC. Samples of human prostate tissue (normal and cancerous) were obtained from patients at Pittsburgh Hospital, by Dr. Melhem from University of Pittsburgh, PA. They were analyzed for stage of the tumor by a pathologist and frozen in OCT (at $-70^{o}C$) for further assays.

RT-PCR analysis of FABP mRNA: Total RNA was isolated from cells and tissue samples using the TRIZOL method (5) according to the manufacturer's (Life Technologies) instructions. RT-PCR analysis for gene expression pattern of the five FABPs (Adipose, Epidermal, Brain, Intestine and Liver) was performed using specific primers for each FABP (see Table 1). The homology of PCR products with the corresponding GENEBANK sequence for each FABP was verified by sequencing the PCR products using a Cycle sequencing kit (Amersham, Arlington Heights, IL).

Quantitation of PCR products: Samples were resolved on agarose gels, scanned and digitized using the NIH-Scion Image program. For each RNA sample, a parallel reaction containing primers for actin and/or S9 ribosomal protein (Clontech, Palo Alto, CA) were performed as internal control for normalization of samples. Each band (digitized value) for the respective FABP was then normalized with the actin/S9 value and then comparison made between prostate normal and cancer cells.

We have also quantitated the PCR products using biotin labeled primer and performed an ELISA assay with amine labeled specific internal probes that were precoated onto 96 well plates (ELISA kits for each specific FABP was obtained from KamTek Inc, MD). The biotinylated PCR products were hybridized with the amine labeled probe and analyzed with Streptavidin coated HRP resulting in a color reaction which is measured at 630nm.

Western Blot Analysis of Liver-FABP proteins: Prostate cells (PrEC, normal immortalized, and DU145 cancer) were grown in their respective media for 48 hrs. The conditioned media from the cells were collected, precipitated with ammonium sulfate, subjected to dialysis in 0.1M Tris, pH 7.5 and quantitated using Bradford Reagents (Bio Rad, Hercules, CA). The western blot was then incubated (5) with primary antibody (polyclonal) for L-FABP (Research & Diagnostics, Cambridge, MA) followed by incubation with HRP-conjugated secondary antibody. Bound L-FABP antibody was detected using an ECL detection kit (Amersham, Arlington Heights, IL).

RESULTS:

Expression pattern of E-, H-, and A-FABP in prostate normal and cancer cells:

Expression levels of various FABP were analyzed upon extraction of RNA from the normal primary cultures of prostate cells, PrEC and compared with that from 2 prostate cancer cell lines,

LNCaP and PC3. We observed a dramatic decrease in the message levels of A-, E and H-FABP in cancer cells when compared to the normal prostate cells (Fig. 1a). For E-FABP, the decrease was 6- to 50-fold. The level of expression of E-FABP was the predominant one when compared with all the other FABP levels in the prostate cells. Similarly, H- and A- FABPs showed a decrease in expression in the cancer cell lines relative to the normal cells. In another prostate cancer cell line, DU 145, the expression of A-FABP was undetectable (data not shown).

Expression of I-, B-, and L-FABP in prostate cells:

I-FABP gene expression was elevated significantly (6- to11-fold) in the cancer cells LNCaP and PC3 when compared to PrEC, the normal prostate cells (Fig. 1b). Additionally, L-FAPB was increased 6-fold in both tumor lines vs the normal cells. B-FABP was elevated 7-fold in LNCaP prostate tumor cells but was completely undetectable in PC3 cancer cell cultures. PC3 cells are androgen receptor negative and highly undifferentiated. In contrast, the androgen receptor positive LNCaP cells are well differentiated. Another androgen receptor negative, undifferentiated cell line, DU 145, showed a pattern similar to PC3 cells in expression of the various FABPs (data not shown).

Expression of FABP in human prostate tissue samples:

Tissue samples were provided from the University of Pittsburgh/VA Medical center and stage analysis had been performed on the samples by them, but that information was blinded until after analysis of the samples for FABP levels. RNA was isolated and RT-PCR performed for the various FABPs. As shown in Fig. 2a & b, there was a significant decrease in the levels of E-FABP (12-fold), H-FABP (5-fold), A-FABP (7-fold). In contrast, L-FABP was elevated 10-fold in tumor vs normal prostate tissue, and I-FABP was approximately 7-fold elevated. B-FABP was unique in that it was highly elevated (13-fold) in moderate and well-differentiated prostate

carcinomas (9 samples) and was undetectable in 2 samples of poorly differentiated prostate carcinoma tissues. As was found with the prostate cancer cell lines, CRABP-I (cellular retinoic acid binding protein) followed the pattern of expression of B-FABP in these 5 normal and 11 staged prostate cancer tissue samples.

Secretion of L-FABP by prostate cancer cells:

Conditioned media from PrEC and DU145 were collected after 48hrs of culture and were concentrated and analyzed by western blots. A 14 kD band was detected by L-FABP antibody only in the cancer cells (Fig. 3). No L-FABP protein was detected in the normal PrEC cells.

DISCUSSION:

There are multiple reports supporting a role for arachidonic acid and its metabolites in the proliferation of prostate cancer cells. In radical prostatectomy specimens, AA turnover was 10 times higher in the tumor as compared to surrounding uninvolved prostate tissue. Diets high in corn oil, a fat rich in the AA precursor linoleic acid, markedly stimulated the growth of human prostate cancer xenografts in nude mice (7, 15, 20). Studies have implicated an association of linoleic acid (LA) with prostate cancer. Linoleic acid is the most prevalent unsaturated fatty acid component of commonly used cooking oils. A large prospective study of American men showed a positive association between linoleic acid in the diet and prostate cancer (8). Moreover, *in vitro* studies of the human prostate cancer cell line PC-3 showed stimulated growth in the presence of linoleic acid whereas the long chain fatty acids may inhibit tumorigenesis (15). Transport and utilization of bioactive lipids in a cell is a critical component in the process by which these molecules modulate proliferation through interactions with nuclear receptors. The familyof

cytoplasmic proteins, known as FABPs, are crucial transporters of bioactive lipids. These proteins may also be important in the regulation of free fatty acid concentrations within the cell. Such crucial participation in the trafficking and availability of fatty acids suggests that the FABPs may be critical links in the mechanistic chain connecting dietary fat with cancer.

FABPs are known to bind many different groups of fatty acids and their derivatives, including eicosanoids and other bioactive lipids [reviewed in (19)] thus suggesting a crucial role in promotion of prostate cancer cell growth. L-FABP exhibits different lipid binding characteristics from that of A-FABP or H-FABP. L-FABP transfected into rat hepatoma cells also mediates cell induction by carcinogenic peroxisome proliferators (12).

Changes in expression of FABPs have been reported for bladder cancer. Psoriasis-associated FABP (E-FABP) was noted to increase in level with increase in differentiation of bladder squamous cell carcinomas (13). Secretion of FABPs have been detected in serum and urine of patients suffering from myocardial infarction (14,16, 18), whereas psoriasis-associated FABP (E-FABP) was among a number of marker proteins detected in the urine of bladder cancer patients (14). In addition, loss of adipocyte-FABP (A-FABP) was reported with progression of human bladder transitional cell carcinomas (2). The presence of A-FABP correlated with the grade and stage of the disease. The A-FABP protein was present in high levels in grade I and II Transitional Cell Carcinomas whereas grade III had 37% reduction and grade IV had no A-FABP expression. A-FABP may act as a growth inhibitor similar to the mammary derived growth inhibitor (MDGI, now identified as being homologous with H-FABP) protein in breast cancer. Loss of A-FABP expression appears to be a prognostic marker for aggressive bladder cancer. In our studies we also observed a significant loss of A-FABP expression in tumor cells when compared to the normal primary cultured cells. The normal cells showed high levels of A-

FABP and E-FABP. The expression of E-FABP was also 6 to 50- fold lower in cancer cells when compared to the normal cells. In human prostate tissue samples we observed a dramatic reduction (ca. 10-fold) of the levels of E-FABP in tumor samples when compared to the normal tissue samples. This shows that the levels of FABP observed in tissue culture prostate cells were confirmed in the human biopsy tissue samples.

The novel finding in this work is that in addition to the decreased heart-type FABPs in the tumor cells, there was a dramatic increase in I- and L-FABPs. L-FABP has been shown to be elevated significantly in metastatic or regenerating liver vs. normal liver (4, 12). In prostate cells we observed increased expression of L-FABP and I-FABP in cancer cells compared to the normal prostate cells. In our studies we report for the first time the secretion of L-FABP protein only from prostate cancer cells and not from normal prostate cells. We propose that the secretion of L-FABP can suggest the presence of cancer in these prostate cells.

In the current study we observed a distinct pattern of gene expression of FABPs in prostate cells. In normal cells or tissue there is a high level of expression of A-FABP and E-FABP that decreases with increase in progression of tumor stage. However the levels of L-FABP and I-FABP are very low or undetectable in normal cells and are found in high levels in cancer cells and tumor samples. Interestingly, B-FABP was not found in normal cells, and was detected in well-differentiated prostate cancer cells and patient biopsy tissues. A-, B-FABP and CRABP-I might be indicators of a more aggressive form of prostate cancer. Most importantly, the pattern of expression of E-, I-, L- and A-FABP clearly delineates tumor from normal cells.

These results suggests that A-FABP and E-FABP may act as a tumor suppressors in prostate cells similar to MDGI (H-FABP) in MCF-7 cells (10). Of the few FABPs tested so far, we have found altered levels of FABPs; individually, each of these have been shown, in other

cell systems, to correlate with the normal or tumor state. Our study is the first report to show concomitant decreases in the heart-type FABPs (A- and E-FABPs) and increases in mitosis promoting FABPs (I-FABP and L-FABP). We suggest that there is a distinct balance between the levels of various FABPs in a cell that might predict the stage and degree of malignancy. B-FABP and CRABP-I may be key and support the notion that level(s) of FABP(s) will correlate with stage of prostate cell proliferation and perhaps aggressive tumors. We therefore suggest that the pattern of expression of the various FABPs may provide information indicating the potential usefulness of FABPs as possible early detection markers, along with PSA levels, and give some meaningful answers in identifying different stages of prostate cancer.

REFERENCES:

1. Bass NM. The cellular fatty acid binding proteins: aspects of structure, regulation and function. Int Rev Cytol., 111:1988.
2. Celis JE, Ostergaard M, Basse B, Celis A, Lauridsen JB, Ratz GP, Andersen I, Hein B, Wolf H, Orntoft TF and Rasmussen HH. Loss of adipocyte-type fatty acid binding protein and other protein biomarkers is associated with progression of human bladder transitional cell carcinomas. Cancer Res., 56:4782-90, 1996.
3. Chaudry AA and Dutta-Roy AK. Purification and characterization of a fatty acid binding protein from human prostatic tissue. Lipids, 28:383-8, 1993.
4. Custer RP and Sorof S. Target polypeptide of a carcinogen is associated with normal mitosis and carcinogen-induced hyperplasia in adult hepatocytes. Proc Natl Acad Sci USA., 81:7638-6742, 1984.
5. Das R and Vonderhaar BK. Transduction of prolactin's growth signal through both long and short forms of the prolactin receptor. Mol Endocrinol., 9:1750-1759, 1995.

6. Franceschi S. Fat and prostate cancer. Epidemiol., 5:271-273, 1994.

7. Ghosh J and Myers EE: Arachidonic acid stimulates prostate cancer cell growth: critical role of 5-lipoxygenase. Biochem Biophys Res Comm., 235:418-423, 1997.

8. Giovannucci E, Rimm EB, Colditz GA, Stampfer MJ, Ascherio A, Chute CC and Willett WC. A prospective study of dietary fat and risk of prostate cancer. J Nat Cancer Inst., 85:1571-1579, 1993.

9. Grosse R, Boehmer FD, Langen P, Kurtz A, Lehmann W, Mieth M and Wallukat G. Purification, biological assay and immunoassay of mammary-derived growth inhibitor. Methods Enzymol., 198:425-440, 1991.

10. Huynh H, Alpert L and Pollak M. Silencing of the mammary-derived growth inhibitor (MDGI) gene in breast neoplasms is associated with epigenetic changes. Cancer Res., 56:4865-70, 1996.

11. Keler T, Barker CS and Sorof S. Specific growth stimulation by linoleic acid in hepatoma cell lines transfected with the target protein of a liver carcinogen. Proc Natl Acad Sci U S A., 89:4830-4, 1992.

12. Khan SH and Sorof S. Liver fatty acid-binding protein: specific mediator of the mitogenesis induced by two classes of carcinogenic peroxisome proliferators. Proc Natl Acad Sci U S A., 91:848-52, 1994.

13. Ostergaard M, Rasmussen HH, Nielsen HV, Vorum H, Orntoft TF, Wolf H and Celis JE. Proteome profiling of bladder squamous cell carcinomas: identification of markers that define their degree of differentiation. Cancer Res., 57:4111-7, 1997.

14. Rasmussen HH, Orntoft TF, Wolf H and Celis JE. Towards a comprehensive database of proteins from the urine of patients with bladder cancer. J Urol., 155:2113-9, 1996.

15. Rose DP and Connolly JM. Effects of fatty acids and eicosanoid synthesis inhibitors on the growth of two human prostate cancer cell lines. Prostate, 1811243-254:1991.

16. Sohmiya K, Tanaka T, Tsuji R, Yoshimoto K, Nakayama Y, Hirota Y, Kawamura K, Matsunaga Y, Nishimura S and Miyazaki H. Plasma and urinary heart-type cytoplasmic fatty acid-binding protein in coronary occlusion and reperfusion induced myocardial injury model. J Mol Cell Cardiol., 25:1413-26, 1993.

17. Tipping E and Ketterer B. The influence of soluble binding proteins on lipophile transport and metabolism in hepatocytes. Biochem J., 195:441-52, 1981.

18. Van Nieuwenhoven FA, Kleine AH, Wodzig WH, Hermens WT, Kragten HA, Maessen JG, Punt CD, Van Dieijen MP, Van der Vusse GJ and Glatz JF. Discrimination between myocardial and skeletal muscle injury by assessment of the plasma ratio of myoglobin over fatty acid-binding protein. Circulation, 92:2848-54, 1995.

19. Veerkamp JH, Peeters RA and Matman RGHJ. Structural and functional features of different types of cytoplasmic fatty acid-binding proteins. Biochim Biophys Acta., 1081:1-24, 1991.

20. Wang U, Corr JG, Thaler HT, Tao Y, Fair WR and Heston WDW. Decreased growth of established human prostate LNCaP tumors in nude mice fed a low-fat diet. J. Nat Cancer Inst., 87:1456-1462, 1995.

TABLE 1

SEQUENCES OF PRIMERS USED TO IDENTIFY EACH SPECIFIC FABP

cDNA	PRIMER	SEQUENCE	ANNEALING TEMP.
A-FABP	FW RV	GAAACTTGTCTCCAGTGAAAAC GGGAGAAAATTACTTGCTTG	55
L-FABP	FW RV	CTCTATTGCCACCATGAGTTTC GCTGATTCTCTTGAAGACAATCTG	50
H-FABP	FW RV	ATTCGGCACGAGGTAGCTTC AGAGGAAGAAATGAGGCAATG	58
I-FABP	FW RV	ACAGCACTTGGAAGGTAGACC TTCTCGGACAGTATTCAGTTCG	52
E-FABP	FW RV	CCGACGCAGACCCCTCTC GATCCGAGTACAGGTGACATTGTTC	56
B-FABP	FW RV	CGCTCCTGTCTCTAAAGAGGGG TGGGCAAGTTGCTTGGAGTAAC	56

FIGURE LEGENDS

Figure 1. Expression of Various FABP in Cultures of Normal and cancerous Prostate Cell Lines. Prostate cells were cultured according to supplier instructions and total RNA isolated using the Trizol method. Equal amounts were taken to determine gene expression levels using the specific primers (Table I) and performing RT-PCR. The PCR product was resolved on a 1% agarose gel and detected with Ethidium bromide and intensities were measured using NIH-Scion Image and normalized to actin and/or S9 ribosomal protein. Figure 1A shows expression of the FABPs associated with normal cells, E-, H-, and A-FABP in PrEC normal prostate cells ( ), and two prostate cancer cell lines, LNCaP ( ), and PC3 ( ). Figure 1B shows expression of the FABPs associated with tumor cells, I-, B-, and L-FABP.

Figure 2. Expression of Various FABP in Normal and Cancerous Prostate Tissue Samples. Trizol was added to tissue samples obtained from patients and the samples were processed and detection carried out as described in the methods. RT-PCR was performed for the indicated FABPs using specific primers. Data represented here are the expression levels of FABPs after normalizing to actin values. Figure 2A shows shows expression of the FABPs associated with normal cells, E-, H-, and A-FABP in tissues from 5 normal prostate biopsy samples ( ), and 11 prostate cancer biopsy specimens ( ). Figure 1B shows expression of the FABPs associated with tumor cells, I-, B-, and L-FABP.

Figure 3. Western Blot Analysis of Liver-FABP proteins: Prostate cells (PrEC and DU145) were grown in their respective media for 48 hrs. The conditioned media from the cells were collected

by centrifugation at 3000xg. The supernatant media were precipitated with saturating levels of ammonium sulfate followed by dialysis in 0.1M Tris-HCl, pH 7.5 and quantitated using Bradford method. Equal amounts of protein were resolved on a SDS-PAGE followed by transfer onto nitrocellulose paper. The blot was then incubated with primary antibody for L-FABP followed by incubation with HRP-conjugated secondary antibody. Bound L-FABP antibody was detected using an ECL detection kit. The band represents L-FABP from the conditioned media in DU-145 cells.

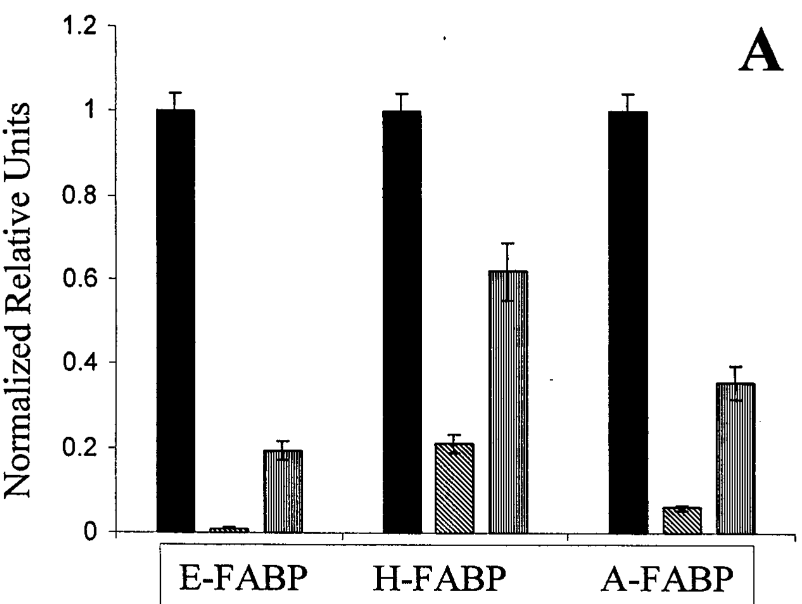
FIGURE LEGENDS

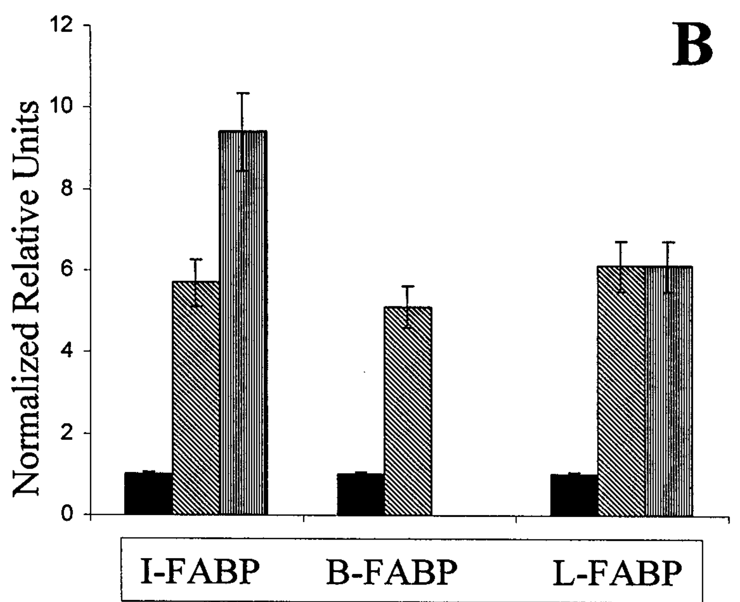
Figure 1. Expression of Various FABP in Cultures of Normal and cancerous Prostate Cell Lines. Prostate cells were cultured according to supplier instructions and total RNA isolated using the Trizol method. Equal amounts were taken to determine gene expression levels using the specific primers (Table I) and performing RT-PCR. The PCR product was resolved on a 1% agarose gel and detected with Ethidium bromide and intensities were measured using NIH-Scion Image and normalized to actin and/or S9 ribosomal protein. Figure 1A shows expression of the FABPs associated with normal cells, E-, H-, and A-FABP in PrEC normal prostate cells (■), and two prostate cancer cell lines, LNCaP (▨), and PC3 (▥). Figure 1B shows expression of the FABPs associated with tumor cells, I-, B-, and L-FABP.

Figure 2. Expression of Various FABP in Normal and Cancerous Prostate Tissue Samples. Trizol was added to tissue samples obtained from patients and the samples were processed and detection carried out as described in the methods. RT-PCR was performed for the indicated FABPs using specific primers. Data represented here are the expression levels of FABPs after normalizing to actin values. Figure 2A shows shows expression of the FABPs associated with normal cells, E-, H-, and A-FABP in tissues from 5 normal prostate biopsy samples (■), and 11 prostate cancer biopsy specimens (▨). Figure 1B shows expression of the FABPs associated with tumor cells, I-, B-, and L-FABP.

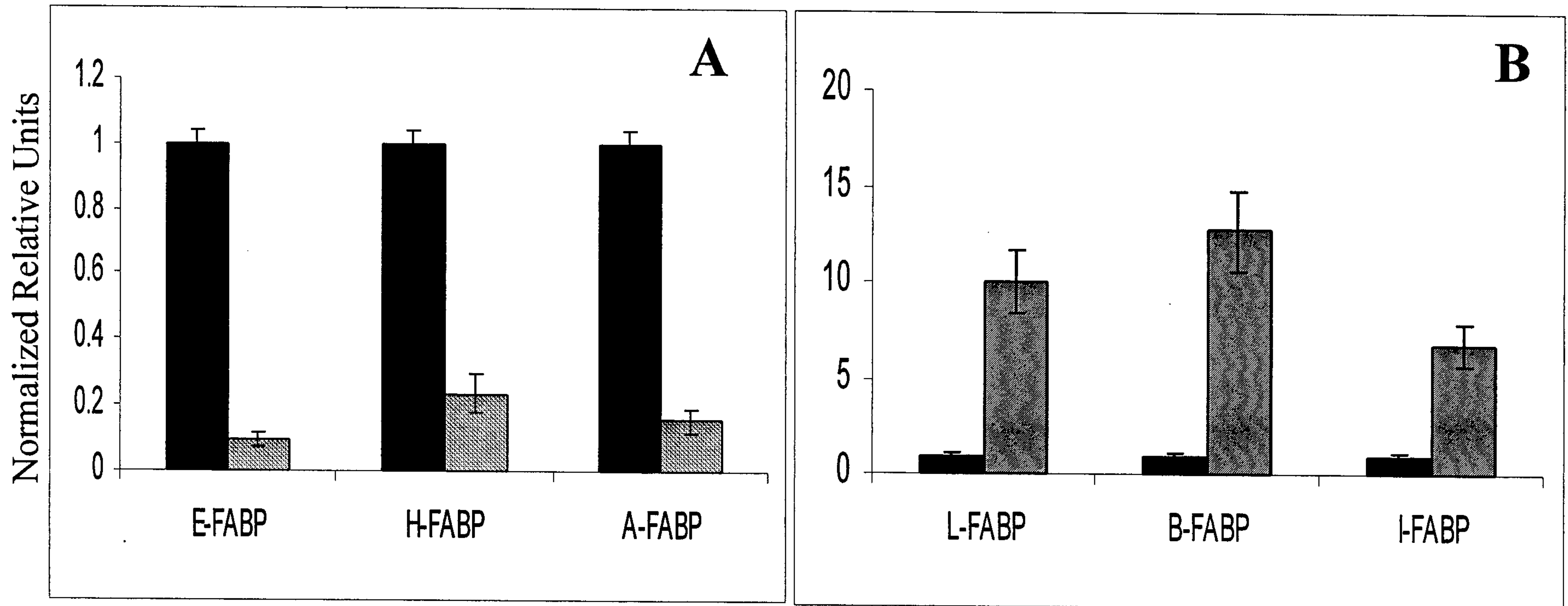
Figure 3. Western Blot Analysis of Liver-FABP proteins: Prostate cells (PrEC and DU145) were grown in their respective media for 48 hrs. The conditioned media from the cells were collected

by centrifugation at 3000xg. The supernatant media were precipitated with saturating levels of ammonium sulfate followed by dialysis in 0.1M Tris-HCl, pH 7.5 and quantitated using Bradford method. Equal amounts of protein were resolved on a SDS-PAGE followed by transfer onto nitrocellulose paper. The blot was then incubated with primary antibody for L-FABP followed by incubation with HRP-conjugated secondary antibody. Bound L-FABP antibody was detected using an ECL detection kit. The band represents L-FABP from the conditioned media in DU-145 cells. Antisera specific to the various FABPs has been difficult to produce, due to the close structural homology that they share.





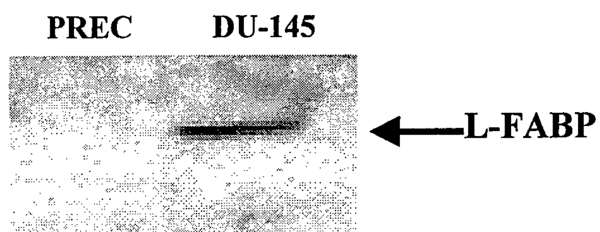
DAS, et al.
Figure 1
TOP OF GRAPH ↑



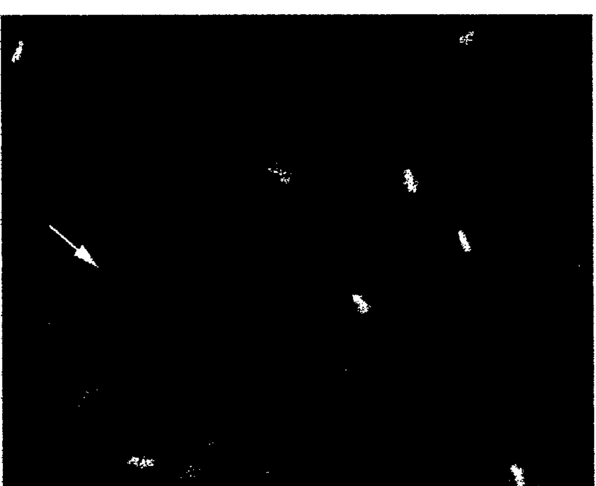
DAS, et al.
Figure 2
TOP OF GRAPH ↑

DAS, et al.
Figure 3

TOP OF GRAPH ↑

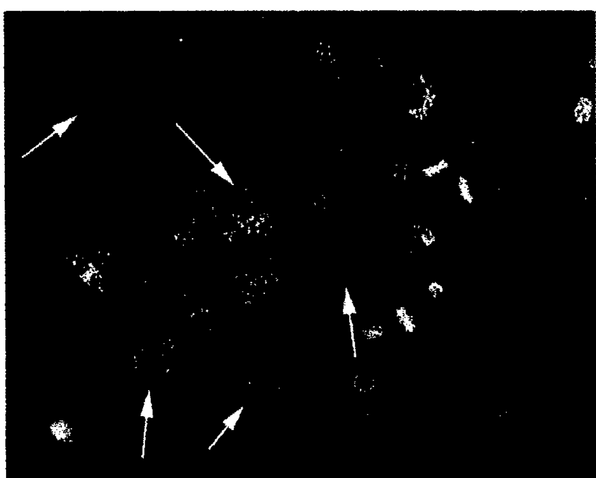


Overexpression of A-FABP Induces Apoptosis in DU-145 Prostate Cancer Cells

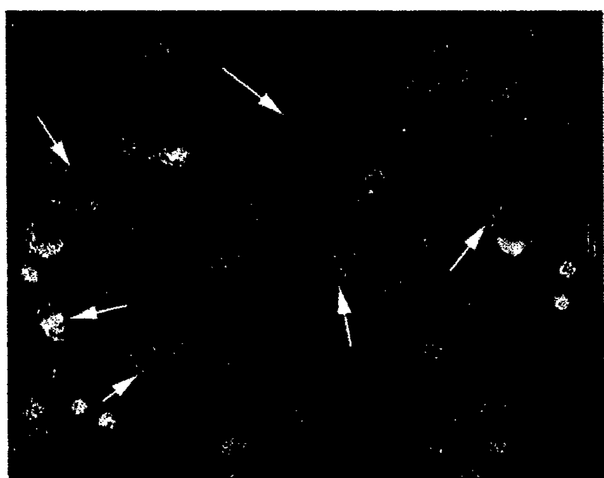


0μM

5μM



10μM



20μM

Figure 8