

# Expression Pattern of Fatty Acid-binding Proteins in Human Normal and Cancer Prostate Cells and Tissues<sup>1</sup>

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## ABSTRACT

**Purpose:** Fatty acid-binding protein (FABP) expression patterns were evaluated as potential markers and therapeutic targets for prostate cancer.

**Experimental design:** FABP expression levels were determined by reverse transcription-PCR in cultured prostate normal and tumor cells and in human biopsy samples. Regulation of cellular processes was examined using FABP antisense constructs.

**Results:** Prostate cells express a variety of different FABPs. Liver (L)- and intestine-FABPs were elevated 5–9-fold in prostate cancer compared with normal primary prostate cells. In contrast, adipose- and epidermal-FABPs were markedly down-regulated (3–20-fold) in cancer *versus* normal cells. Similar expression patterns were found in human tissue biopsy samples. However, brain-FABP had a distinct pattern of expression: it was overexpressed only in LNCaP cells and in well-differentiated tissue samples, suggesting a stage-specific expression profile. Secretion of L-FABP protein was observed from DU 145 prostate cancer cells, but not in the culture fluid of normal prostate epithelial cells. Antisense oligodeoxynucleotides, designed to block production of epidermal-FABP (a marker for normal prostate cells), caused increased proliferation in DU 145 prostate cancer cells. In vivid contrast, antisense oligodeoxynucleotides to L-FABP (overexpressed in prostate cancer) decreased proliferation and caused apoptosis.

**Conclusions:** We propose that there is a distinct balance between these groups of FABPs, whose altered regulation in cells may play a role in prostate cancer. Furthermore, the pattern of expression and secretion of FABPs have the po-

tential to serve as a diagnostic marker for an 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, lipid metabolism, and the development of prostate cancer (1–5). Linoleic acid, a common component of dietary fat, and its metabolic derivative, AA,<sup>3</sup> have both been associated with prostate tumor cell proliferation (2, 4). 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. A family of cytoplasmic proteins known as FABPs mediates transport and utilization of such lipids. Several studies suggest that FABPs increase the solubility of fatty acids in cell cytoplasm, causing a net diffusion of fatty acids from the plasma membrane to the intracellular membrane compartments (6).

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  $M_r$ 14,000 in size, encoding about 115 amino acids (7). The designations for each of the FABPs has been derived from the tissue from which it was originally isolated and includes: (a) adipocyte (A-FABP); (b) heart or muscle (H-FABP); (c) brain (B-FABP); (d) epidermis or psoriasis-associated (E-FABP); (e) liver (L-FABP); (f) intestine (I-FABP); and (g) myelin or P2 (P2-FABP). As a group, human A-FABP, H-FABP, B-FABP, and E-FABP share between 50 and 65% protein sequence homology and contain a tyrosine near residue 20 that can be phosphorylated (7, 8). Although they are intracellular proteins, specific FABPs have been detected in elevated levels in plasma or urine of patients suffering from myocardial infarction or bladder cancer (9–11). FABPs are known to bind many different groups of fatty acids and their derivatives, including eicosanoids and other bioactive lipids (reviewed in Refs. 8 and 12). L-FABP exhibits different lipid-binding characteristics from that of A-FABP or H-FABP. Direct comparisons of their ability to bind DNA or carcinogens have not been studied.

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

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<sup>3</sup> The abbreviations used are: AA, arachidonic acid; FABP, fatty acid-binding proteins; ODN, oligodeoxynucleotide; MDGI, mammary-derived growth inhibitor; RT-PCR, reverse-transcription-PCR; HRP, horseradish peroxidase; LA, linoleic acid; PrEC, prostate epithelial cells; CRABP, cytosolic retinoic acid-binding protein; PPARs, peroxisome proliferator-activated receptors.

(13). Transfection of L-FABP into hepatoma cells increased their proliferation (14, 15). In contrast, MDGI (also known as H-FABP) appears only in normal lactating cells and not in tumor mammary cells (16). Transfection of a cDNA clone of MDGI into breast cancer cells results in the loss of tumorigenicity (17). Other FABP types have been implicated in control of cell proliferation or cancer (18).

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 (19), suggesting L-FABP may be expressed in these cancer cells. However additional studies to define further FABPs in prostate normal or cancer cells have not been reported.

In this study, we have examined the expression levels of FABPs in several prostate normal and cancer cell lines to establish a correlation between the presence and levels of the various FABPs with the stage of cancer represented by each cell line. The levels of expression of mRNA for six 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 pattern of expression is altered in prostate cancer when compared with normal cells. A- and E-FABP (heart type) were down-regulated in cancer cells, whereas the liver types (L-FABP and I-FABP) were up-regulated in cancer cells. Brain (B-FABP), structurally related to heart-type FABPs, was elevated only in certain states of differentiated prostate cancer cells. Similar patterns for FABP expression were found in clinical samples of human prostate biopsy samples. We have also established that when overexpressed, these FABP proteins are secreted from prostate cancer cells into the media in tissue culture. To understand the mechanism of FABP action we have used antisense ODNs to either E-FABP or L-FABP to study responses in DU 145 cells and have shown that FABPs play a direct role in cell proliferation and apoptosis. Thus, these FABP profiles 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, Inc. (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 were followed as described in the manufacturers' instructions (American Type Culture Collection, McLean, VA, and Clonetics Corp., San Diego, CA, respectively) and each cancer cell line was cultured in the appropriate culture fluid containing 5% fetal bovine serum. Primary cultures of normal prostate cells (PrEC) were obtained from Clonetics Corp. and cultured in the fluid formulated especially for these primary cell cultures. In one experiment, DU 145 was cultured in the PrEC media to determine whether that specialized culture fluid, designed especially for primary cell cultures, would influence FABP expression patterns in the tumor cells. The pros-

tate immortalized and cancer cell lines PZ-HPV-7, LNCaP, PC-3, and DU 145 were obtained from American Type Culture Collection. Samples of human prostate tissue (normal and cancerous) were resected, and total prostatectomy specimens were obtained from patients at the Veterans Administration Medical Center from the University of Pittsburgh, Pittsburgh, PA, by Dr. Mona Melhem.

Tissue samples from human prostate were surgically removed at the Veterans Administration Medical Center-Pittsburgh. Tissue microdissection was done at two levels. (a) At the gross pathology level, prostate specimens were examined fresh and any suspicious lesions cut out by a scalpel blade. This tissue was then frozen in OCT (Sakura, Terrence, CA), and a frozen 5- $\mu$ m section cut and stained with H&E to assure the presence of tumor. Adjacent tissue was also submitted for paraffin-embedded diagnostic sections. This is routinely done in Dr. Mona Melhem's lab; and (b) for FABP analysis, the tissue was microdissected into normal tissue *versus* foci of tumors, RNA was extracted; and RT-PCR was performed to determine the levels of selected FABPs using these matched pairs of normal/tumor samples.

**RT-PCR Analysis of FABP mRNA.** Tissues from patients were preserved by embedding in OCT blocks. Total RNA was isolated from cells and tissue samples using the TRIZOL method (20) according to the manufacturer's instructions (Life Technologies, Inc.) with some modifications. The RNA samples were treated with DNase-1, to remove genomic DNA, and reprecipitated. RT-PCR analysis for gene expression pattern of the seven FABPs (adipose, epidermal, heart, brain, intestine, myelin, and liver) was performed using specific primers for each FABP (see Table 1). The homology of PCR products with the corresponding GenBank sequence for each FABP was verified by sequencing the PCR products using a Cycle sequencing kit (Amersham, Arlington Heights, IL).

**Quantitation of PCR Products.** Different numbers of PCR cycles were used to determine the cycle number at the linear range of PCR products. That was determined to be 30 cycles for the *FABP* genes and 28 cycles for the housekeeping genes. Equal amounts of RNA samples were used to perform the RT-PCR reaction for all of the genes. Samples were resolved on agarose gels, scanned, and digitized using a Multifluor FX scanner and Quantity One program (Bio-Rad, Hercules, CA). For each RNA sample, a parallel reaction containing primers for actin and/or S9 ribosomal protein (Clontech, Palo Alto, CA) was performed as an internal control for normalization of samples.

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 were obtained from KamTek Inc., Rockville, MD). The biotinylated PCR products were hybridized with the amine-labeled probe and analyzed with streptavidin-coated HRP, resulting in a color reaction that was measured at 630 nm. This method of quantitating PCR products is much more sensitive than ethidium bromide-stained gels.

**Western Blot Analysis of L-FABP Proteins.** Prostate cells (PrEC, normal, and DU 145, cancer) were grown in their respective media for 48 h. The conditioned media from the cells were collected, precipitated with ammonium sulfate, dialyzed in

Table 1 Sequences of primers used to identify each specific FABP

The sequence of primers and annealing temperatures were designed for specific recognition of each indicated FABP.

cDNA	Primer	Sequence	Annealing temperature	Accession no.
A-FABP	74–95 564–543	GAAACTTGTCTCCAGTGAAAAC GGGAGAAAATTACTTGCTTG	55	J02874
L-FABP	29–51 414–394	CTCTATTGCCACCATGAGTTTC GCTGATTCTCTTGAAGACAATCTG	50	M10050
H-FABP	3–22 529–507	ATTCGGCACGAGGTAGCTTC AGAGGAAGAAAATGAGGCAATG	58	X56549
I-FABP	72–92 385–364	ACAGCACTTGAAGGTAGACC TTCTCGGACAGTATTCAGTTCC	52	M18079
E-FABP	4–22 438–414	CCGACGCAGACCCCTCTC GATCCGAGTACAGGTGACATTGTTC	56	M94856
B-FABP	44–65 639–618	CGCTCCTGTCTCTAAAGAGGGG TGGCAAGTTGCTTGGAGTAAC	56	D88648
Myelin-FABP	37–53 430–408	GAGCAACAAATTCCTGGC GACCTTCTCATAGATTCTGGTGC	54	D16181

0.1 M Tris (pH 7.5), and quantitated using Bradford Reagents (Bio-Rad). Concentrated media alone was used as a control in one of the lanes. Proteins were resolved on SDS-PAGE, and the Western blot (20) was incubated with primary antibody (polyclonal) for L-FABP (Research Diagnostics, Cambridge, MA) and then with HRP-conjugated secondary antibody. Bound L-FABP antibody was detected using an enhanced chemiluminescence detection kit (Amersham, Arlington Heights, IL).

**Apoptosis Study of L-FABP Antisense ODN.** DU 145 cells were plated overnight on an eight-well chamber slide. The next day, 10  $\mu$ M L-FABP antisense or sense ODNs were added, and they were incubated for 48 h. The cells were then fixed with paraformaldehyde and stained with Hoechst 33258 dye for 30 min. Cells with bright, fragmented, condensed nuclei were identified as apoptotic cells. The number of apoptotic cells was counted in 10 microscopic fields ( $\times 40$ ) in each case.

**DNA Fragmentation Assay.** DNA fragmentation study was carried out using the apoptotic DNA ladder kit (Roche Molecular Biochemicals, Indianapolis, IN). Briefly,  $2 \times 10^6$  cells were suspended in 200  $\mu$ l of PBS, and the binding buffer was added to a final volume of 400  $\mu$ l. The binding mixture was incubated for 10 min at room temperature and 100  $\mu$ l isopropanol was added. The cell lysate was applied on a filter tube, washed twice, and DNA was eluted using a prewarmed (70°C) elution buffer. The DNA samples were subjected to electrophoresis using 1% agarose gel containing ethidium bromide.

**Cell Proliferation Study.** Cell proliferation assay was carried out using the CyQUANT kit (Molecular Probes, Eugene, OR), which measures the total amount of DNA present using a propidium iodide formulation. Cells were plated into 96-well microtiter plates. The next day different concentrations of the sense or antisense ODN complementary to L-FABP or E-FABP were added to the cells for different time periods. After 3 days, plates were washed with PBS and stored at  $-70^\circ\text{C}$  overnight. The next day, the plates were thawed at room temperature, and the CyQUANT-GR dye/cell lysis buffer was added to each well. There were negative controls included in the assay to account for baseline fluorescence with the antisense alone with the CyQUANT dye; this value was deducted from all of the values and the actual fluorescent units obtained. The sample fluores-

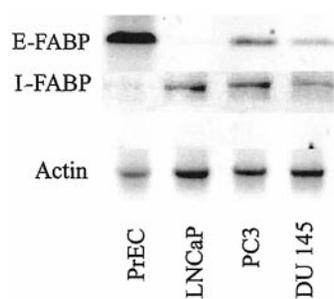
cence was measured using a fluorescence microplate reader at 520 nm ( $\lambda_{\text{ex}} = 480$  nm).

**Statistics.** Significance of difference between samples was determined using Student's paired *t* test.  $P < 0.05$  was regarded as significant.

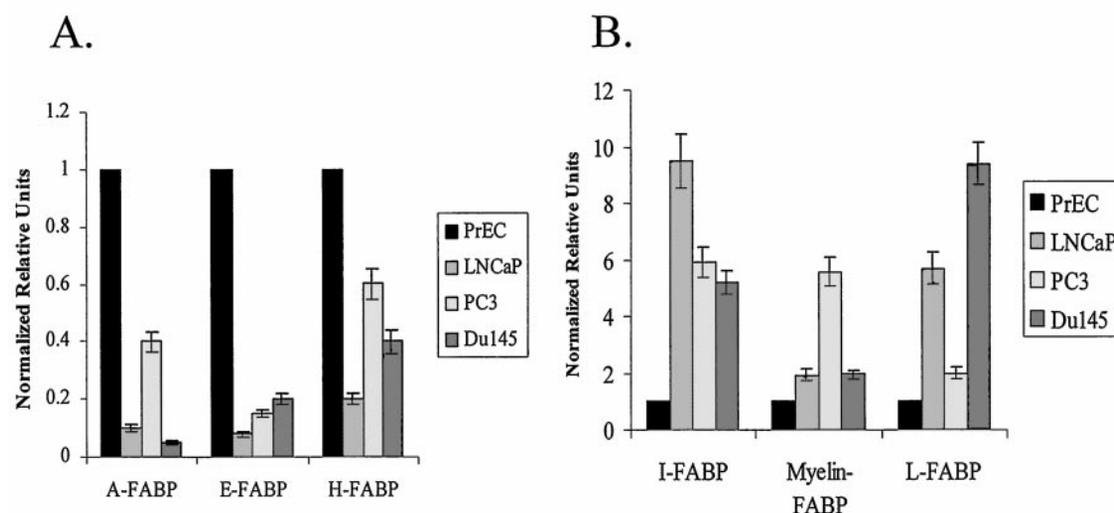
## RESULTS

**Expression Pattern of A-, E-, and H-FABP in Prostate Normal and Cancer Cells.** Expression levels of various FABP were analyzed by RT-PCR from the normal primary cultures, PrEC, and compared with prostate cancer cell lines LNCaP, PC-3 and DU 145. We observed a dramatic decrease in the message levels of A-, E- and H-FABP in cancer cells when compared with the normal prostate cells (Fig. 1A). We have also used an immortalized normal prostate cell line PZ-HPV7 from American Type Culture Collection to measure the normal levels of these FABPs. A-, E-, and H-FABP were similar to the levels observed in PrEC cells. For E-FABP, the decrease was 6–20-fold in the cancer cells. Similarly, A- and H-FABPs showed a significant decrease in expression in the cancer cell lines relative to the normal cells. PC-3, an example of a highly aggressive prostate cancer cell line, showed higher levels of H- and A-FABP compared with the other two cancer cell lines. In DU 145, the expression of A-FABP was very low when compared with the other cancer cell lines. The level of expression of E-FABP was the predominant one in all prostate cells when compared with all of the other FABP.

**Expression of I-, Myelin, and L-FABP in Prostate Cells.** Gene expression of I-FABP was elevated significantly (6–10-fold) in all of the cancer cells LNCaP, DU 145, and PC-3 when compared with PrEC, the normal prostate cells (Fig. 1B). Additionally, L-FABP expression was increased 6-fold in both the cancer lines *versus* the normal cells. L-FABP was elevated 7-fold in LNCaP and 10-fold in DU 145 prostate cancer cells but was comparatively low in PC-3 cancer cell cultures. PC-3 cells are androgen receptor-negative and highly undifferentiated. In contrast, the androgen receptor-positive LNCaP cells are well differentiated. DU 145 showed highest expression of L-FABP and has been characterized as a highly metastatic cell line.



*Fig. 1* Expression of various FABPs in cultures of normal and cancerous prostate cell lines. Prostate cells were cultured, and total RNA was isolated using the Trizol method. Equal amounts were taken to determine gene expression levels using the specific primers (Table 1) and performing RT-PCR. The PCR product was resolved on a 1% agarose gel, and intensities were measured using NIH-Scion Image and normalized to actin and/or S9 ribosomal protein. Also FABP-specific ELISA was used to quantitate the results. The gel picture has been shown for two FABPs, indicating the FABP band after PCR. A, expression of E-, H-, and A-FABP in normal prostate PrEC cells (■), and three prostate cancer cell lines: LNCaP (□), DU 145 (■), and PC-3 (□). B, expression of I-, myelin-, and L-FABP, which are up-regulated only in tumor cells. Use of PrEC culture fluid for DU 145 cancer cells did not alter the FABP expression pattern.

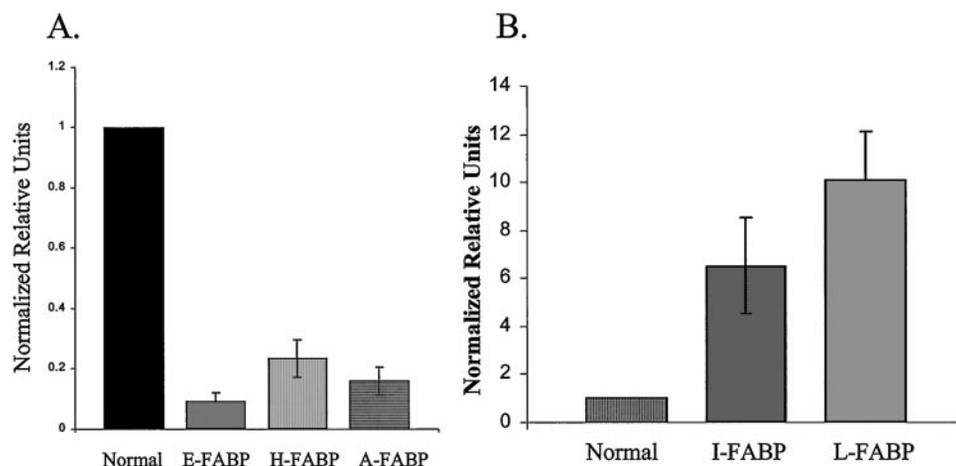


RT-PCR of another member of the FABP family, myelin-FABP, showed up-regulation (6-fold) only in PC-3 cells but showed no change in expression levels in the other two cancer cell lines when compared with the normal PrEC cells.

**Expression of FABP in Human Prostate Tissue Samples.** Tissue samples were provided by the University of Pittsburgh/Veterans Administration Medical Center, and stage analyses were performed on the samples by them, but that information was blinded until after analysis of the samples for FABP levels. The specimens were frozen in OCT and 5- $\mu$ m sections were cut on the cryostat, stained with H&E, and examined by the pathologist before processing. At least 80% of all of the cells within the section were of epithelial prostatic origin and either benign in the benign tissues or malignant in the tumor section to be considered adequate for processing and additional examination. RNA was isolated from these tissues, and RT-PCR was performed for the various FABPs. As shown in Fig. 2A, there was a significant decrease in the levels of E-FABP (12-fold), H-FABP (5-fold), and A-FABP (7-fold) in the tumor biopsies when compared with the normal tissue samples. In contrast, L-FABP was elevated 10-fold and I-FABP was elevated ~7-fold in the tumor samples when compared with the normal prostate tissue (Fig. 2B). These expression profiles of FABPs were very similar to the levels of these proteins found in cultures of prostate cells, indicating the relevance of the expression profiles of these FABPs in differentiating normal from cancer cells.

**Expression Pattern of B-FABP in Cancer Cells and Tissue Samples.** FABP expression of the previously described six genes followed a distinct pattern in normal and cancer cells, however B-FABP showed a very different expression profile. When the levels of B-FABP were analyzed in all of the four cell lines, only LNCaP showed high expression of B-FABP compared with the normal PrEC cells (Fig. 3). There was no B-FABP detectable in either PC-3 or DU 145 cells, suggesting a distinct profile of expression. Upon examination of tissue samples, this was observed again. B-FABP was unique in that it was highly elevated (13-fold) in moderate and well-differentiated prostate carcinomas (nine samples) and undetectable in two samples of poorly differentiated prostate carcinoma tissue. These specimens were examined by a pathologist and the degree of differentiation was diagnosed based on Gleason's classification of prostate cancers (21–23). This scoring system, which takes into consideration the degree of glandular differentiation and cellular atypia, allows a score of 1–10 for each tumor diagnosed. A tumor with a score of 1–4 is considered well differentiated, one with a score of 5–7 is considered moderately differentiated, and one with a score of 8–10 is considered poorly differentiated. Thus, B-FABP might act as a marker for stage-specific analysis in prostate cancer.

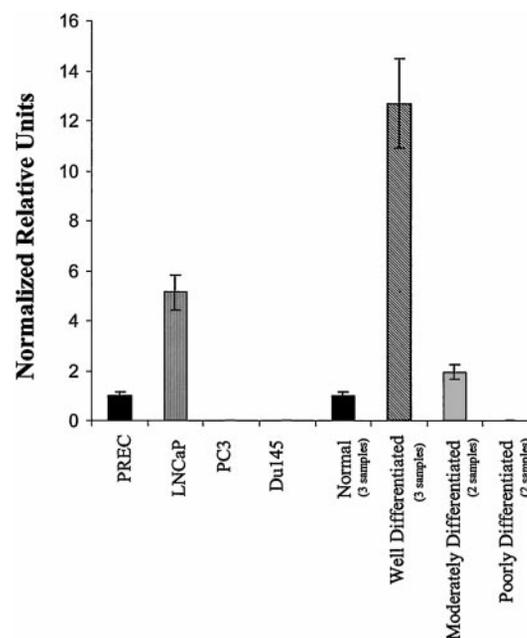
**Secretion of L-FABP by Prostate Cancer Cells.** There are reports that H-FABP was secreted in the serum of patients with myocardial infarction. To determine whether L-FABP,



**Fig. 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 as described in "Materials and 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/S9* values. **A**, expression of the E- (■), H- (▨), and A-FABP (▩) in tissues from five normal prostate biopsy samples (■), and 11 prostate cancer biopsy specimens. The *P*s are 0.017, 0.039, and 0.042 for the three FABPs, respectively. **B**, expression of I-FABP (■) and L-FABP (▩), which are associated with tumor cells. The *P*s are 0.037 and 0.042 respectively.

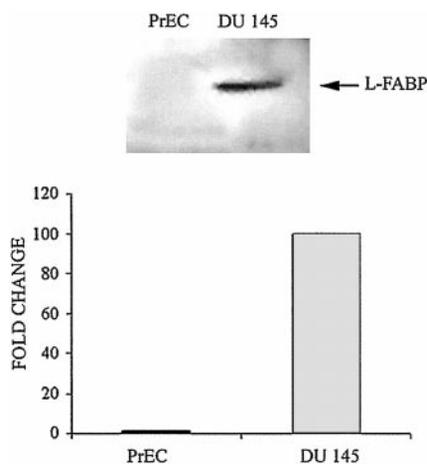
when overexpressed, was secreted into the media by DU 145 cells, conditioned media from PrEC and DU 145 were collected after 48 h of culture, concentrated, and analyzed by Western blots. A  $M_r$  14,000 band was detected by L-FABP antibody only in DU 145 cancer cells (Fig. 4). No L-FABP protein was detected in the normal PrEC cells or in the concentrated media alone used for DU 145 cells. The secretion of these proteins opens up new possibilities of measuring these proteins in body fluids.

**Induction of Apoptosis/Proliferation by L-FABP or E-FABP Antisense ODN Constructs.** The property of FABPs that was first observed was their ability to bind lipids, and a great deal of research has focused on that characteristic. However, these proteins can also bind carcinogens and hormones, and the latter property may relate to a functional role of FABP action in prostate cells. To study the mechanism of their action in prostate cancer cells, we decided to modulate the expression of L-FABP and E-FABP in DU 145 cells. Because DU 145 express high levels of L-FABP, we used a specific antisense ODN complementary to L-FABP and compared it with the sense ODN in these prostate cancer cells. Likewise, E-FABP was the predominant FABP in PrEC normal prostate cells. Using DU 145 prostate cancer cells, antisense to either L- or E-FABPs inhibited expression of the corresponding gene, whereas the sense constructs did not alter any properties investigated (Table 2). For L-FABP, a 6-fold increase in apoptotic cells (after 48 h of exposure to the antisense ODN) was observed, however, there was no significant change with the sense ODN (Table 2 and Fig. 5 *A* and *B*). Similar results were obtained with PC-3 cells exposed to the L-antisense after staining for apoptotic cells. In these experiments, antisense ODN-labeled with fluorescent dye was used to monitor the entry of the oligonucleotide inside the cell. Comparison of the genomic DNA showed multiple smaller DNA fragments in L-FABP antisense-treated cells upon comparison with cultures treated with L-FABP sense (Fig. 5*C*). Proliferation studies in the pres-



**Fig. 3** Expression of B-FABP in cancer cells and tissue samples. RT-PCR was performed for B-FABP using specific primers in prostate cells and tissue samples. Data represented here are the expression levels after normalizing to *actin/S9* genes. For tissue samples, the stage of the cancer was revealed after the analysis of the FABP levels. Ratio of FABP:*actin*, 0.072 in normal tissues; 0.914 in well-differentiated tissues. The number of tissue samples used in each stage of prostate cancer is indicated with each column. The *P*s for well-, moderately, and poorly differentiated samples are 0.038, 0.049, and 0.032, respectively.

ence of antisense ODN to L-FABP showed a significant decrease by 75% in proliferation of DU 145 cells, suggesting a direct role of these FABPs in the regulation of cell proliferation and apoptosis (Fig. 6*A*). When cells were grown in the presence



**Fig. 4** A, Western blot analysis of L-FABP proteins. Prostate cells (PrEC and DU 145) were grown in their respective media. The conditioned media from the cells were collected and precipitated with saturating levels of ammonium sulfate with subsequent dialysis in 0.1 M Tris-HCl (pH 7.5) and quantitated using the Bradford method. Equal amounts of protein were resolved on a SDS-PAGE and then transferred onto nitrocellulose paper. The blot was then incubated with L-FABP with subsequent incubation with HRP-conjugated secondary antibody. Bound L-FABP antibody was detected using an enhanced chemiluminescence detection kit. The band represents L-FABP from the conditioned media in DU 145 cells. Media collected from normal PrEC cells did not show any L-FABP band. B, quantitative measurements of these bands have been determined by using the Bio-Rad Scanner, and the quantity-one image software to digitize the bands.

of antisense to E-FABP, we found the opposite effect on cell proliferation (Fig. 6B). There was a concentration-dependent increase (up to 230%) in proliferation of DU 145 cells when grown in the presence of antisense to E-FABP (Fig. 6B). Thus, by blocking the expression of L-FABP, we observed inhibition of cell proliferation; however, by blocking the expression of E-FABP we increased the proliferation of DU 145 cells.

DU 145 cells express very low levels of A-FABP. In a previous study, we transfected these cells with A-FABP in an inducible vector. Upon induction of A-FABP in DU 145 cells, we observed an increase in apoptosis and inhibition of proliferation (data not shown).<sup>4</sup>

Either by decreasing the expression of L-FABP or increasing the expression of A-FABP in DU 145 cells, we were able to block proliferation, suggesting an important role of FABP in the regulation of cell growth. In addition, further decreasing E-FABP in tumor cells caused a dramatic increase in the proliferation of DU 145 cells. These data show that manipulations of relative ratios of FABPs in tumor cells can modulate proliferation in these cells.

## DISCUSSION

There is a critical need for identifying discriminating diagnostic markers for early detection of cancer. In this study we

**Table 2** Effect of antisense ODN complementary to FABP on apoptosis of DU 145 cells

Antisense ODN sequences were designed to block production of the designated FABP. A control was carried out using the sense construct of L-FABP. Uptake of the constructs was determined using fluorescent dye-labeled ODN. Apoptotic cells were measured using Hoechst staining of treated and untreated cells on chambered slides.

Conditions	% uptake	% apoptotic cells	Down-regulation of E-FABP expression	Down-regulation of L-FABP expression
Control DU 145	N/D <sup>a</sup>	4	N/A <sup>b</sup>	N/A
DU 145 + E-FABP antisense ODN	N/D	5	55%	0
DU 145 + L-FABP sense ODN	35	5	0	0
DU 145 + L-FABP antisense ODN	32	30	0	45%

<sup>a</sup> Not determined.

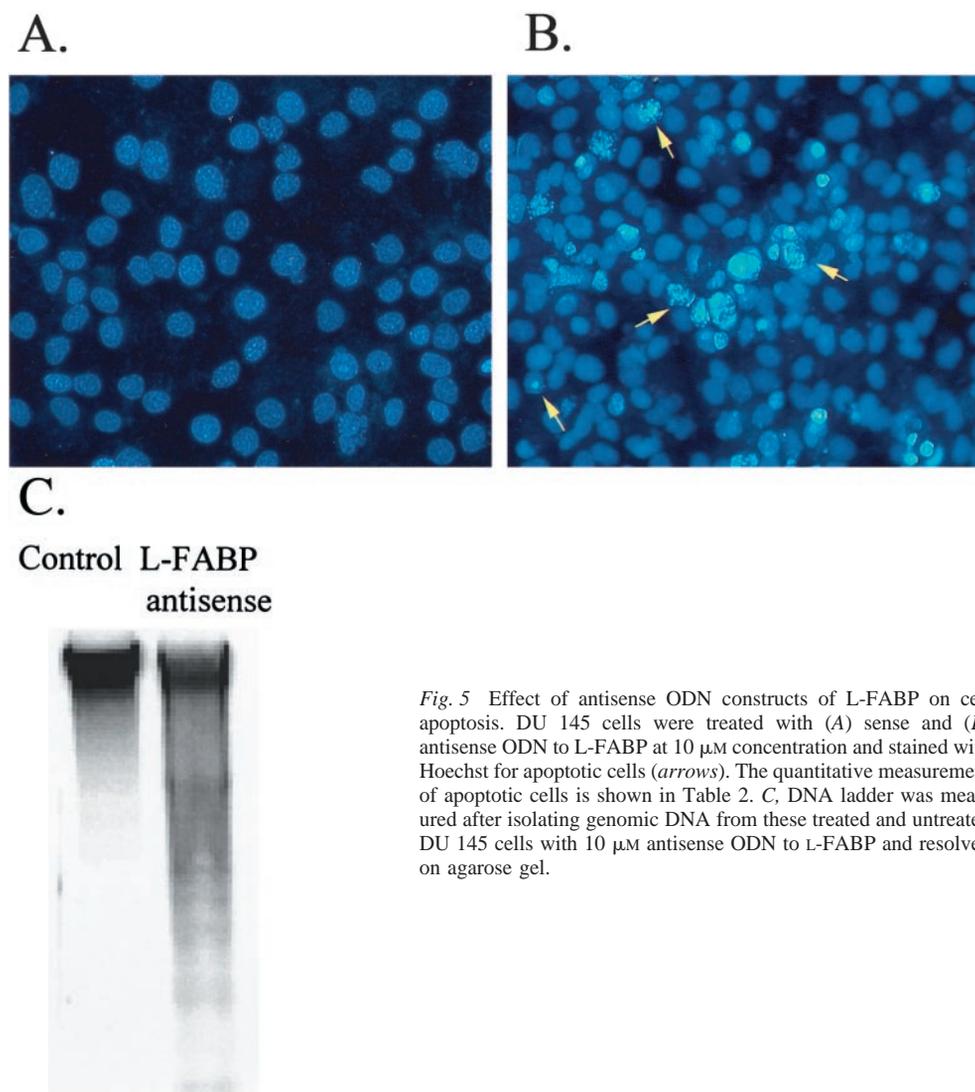
<sup>b</sup> Not applicable.

have shown that individual prostate cell lines and tissues express varying levels of seven different FABPs. There is a distinct pattern of FABP expression that correlates with normal/tumor status of the cells or tissues. Altering this pattern in a number of different ways caused the cells to respond correspondingly. In general, a trend was found in prostate cancer for the FABPs to fall into three groups: FABPs associated with (a) normal prostate; (b) cancerous prostate; or (c) a series of FABPs that may give indications of stage or aggressiveness of a tumor. This observation led us to question what is known about the differences in these various FABPs. As is obvious from their name, they were first characterized as to their ability to bind lipids.

We tested the functional activity of FABP in prostate cell lines ranging from normal epithelial cells (PrEC) to hormone-dependent LNCaP to hormone-independent DU145 and PC-3. These PrEC cells have been routinely used as normal prostate cells to identify differences with various established tumor lines for a variety of studies (24, 25). For normal prostate cells, we have also examined an immortalized cell line PZ-HPV-7 from American Type Culture Collection and found similar levels of FABP expression as with the PrEC cells. The results obtained from these PrEC have been confirmed further using human prostatic normal tissue samples.

There are multiple reports supporting the role of fats, bioactive lipids, and their metabolites in the proliferation of prostate cancer cells. In radical prostatectomy specimens, AA turnover was 10 times higher in the tumor as compared with 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 (2, 5). Studies have implicated an association of LA with prostate cancer. LA 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 LA in the diet and prostate cancer (3). Moreover, *in vitro* studies of the human prostate cancer cell line PC-3 showed stimulated growth in the presence of LA, whereas the long-chain fatty acids may inhibit tumorigenesis (4). Transport and utilization of bioactive

<sup>4</sup> M. DeSantis, R. Das, M. Jett. Adipocyte-fatty acid-binding protein induces apoptosis in Du 145 prostate cancer cells, submitted for publication.



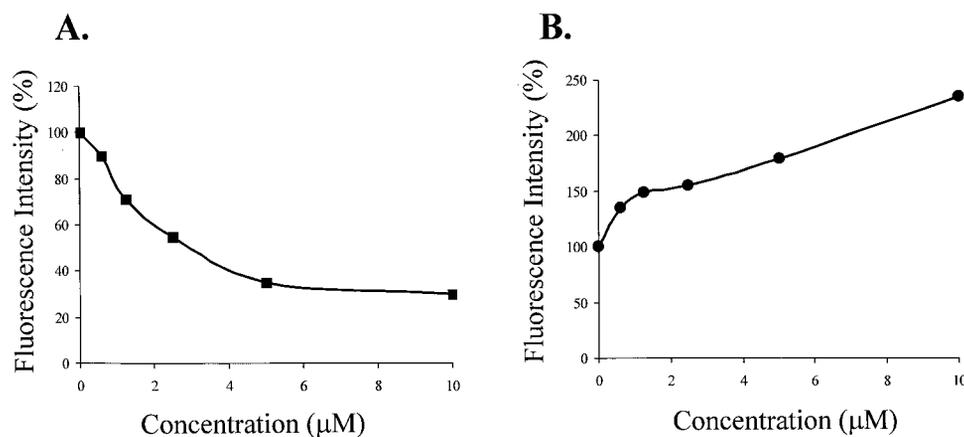
*Fig. 5* Effect of antisense ODN constructs of L-FABP on cell apoptosis. DU 145 cells were treated with (A) sense and (B) antisense ODN to L-FABP at 10  $\mu\text{M}$  concentration and stained with Hoechst for apoptotic cells (arrows). The quantitative measurement of apoptotic cells is shown in Table 2. C, DNA ladder was measured after isolating genomic DNA from these treated and untreated DU 145 cells with 10  $\mu\text{M}$  antisense ODN to L-FABP and resolved on agarose gel.

lipids in a cell is a critical component in the process by which these molecules modulate proliferation through interactions with nuclear receptors. As a family, the cytoplasmic FABPs have been thought to be transporters of bioactive lipids. These proteins may also be important in the regulation of free fatty acid concentrations within the cell and that could be a link in the mechanistic chain connecting dietary fat with cancer.

An example relates to lines of evidence implicating the involvement of arachidonate 12-lipoxygenase, an enzyme metabolizing arachidonic acid to form 12(S)-hydroxyeicosatetraenoic acid, in prostate cancer progression. First, as prostate cancer reaches a more advanced stage, the level of 12-lipoxygenase expression is increased. Second, overexpression of 12-lipoxygenase in human prostate cancer cells stimulates angiogenesis and tumor growth. Third, an inhibitor of 12-lipoxygenase has been found effective against metastatic prostate tumor growth, and the inhibition of 12-lipoxygenase is related with the reduction of tumor angiogenesis. Collectively,

these studies suggest that 12-lipoxygenase regulates tumor angiogenesis in prostate cancer (26).

FABPs are known to bind many different groups of fatty acids and their derivatives, including 12(S)-hydroxyeicosatetraenoic acids, numerous specific eicosanoids and other bioactive lipids (reviewed in Ref. 12), thus suggesting a possible involvement in the promotion of cancer cell growth. Recently Veerkamp *et al.* (8) studied the interactions of various ligands with six different FABPs. They reported that L-, I-, H-, and myelin FABP each had a higher affinity for oleic acid than A-FABP, whereas L- and I-FABP showed a high affinity for 11-dansylamino-undecanoic acid in contrast to the other FABPs. Another report suggested (27) that the binding mode of ligands in E-FABP is identical to that observed in A-FABP, H-FABP, and myelin-FABP. However the binding mode of fatty acids to I-FABP and L-FABP is very distinct from the E-FABP-type binding. L-FABP exhibits different lipid-binding characteristics from that of A-FABP or H-FABP (27). The differences in



**Fig. 6** Effect of L-FABP antisense ODN on proliferation of DU 145 cells. **A**, cells were plated in replicates of six overnight into 96-well microtiter plates. The next day, different concentrations of L-FABP antisense ODN were added to the cells, and samples were measured after 48 h using a fluorescence microplate reader at 520 nm ( $\lambda_{\text{ex}} = 480$  nm). **B**, effect of E-FABP antisense ODN on the proliferation of DU 145 cells. Cells were plated in replicates of six overnight into 96-well microtiter plates. The next day, different concentrations of E-FABP antisense ODN were added to the cells, and samples were measured after 48 h using a fluorescence microplate reader at 520 nm ( $\lambda_{\text{ex}} = 480$  nm). For each study, background fluorescence associated with the presence of ODN was subtracted.

lipid-binding characteristics identified previously do not show direct correlations with the patterns of FABP groups that we have characterized.

Interestingly, FABPs do not bind only lipids, but they avidly bind carcinogens (15, 28), hormones (29–32), and sterols (33). We are continuing to investigate the role of hormones and possible differences in functioning capabilities of FABP from each of the three groupings we have identified. The related molecules, CRABP and PPARs, have been thoroughly characterized as to their ability to bind specific response elements (34) and to alter the expression levels of FABPs.

Other studies have found changes in specific FABPs for various illnesses. Psoriasis-associated FABP (E-FABP) was noted to increase in level with an increase in differentiation of bladder squamous cell carcinomas (35). Secretion of FABPs have been detected in serum and urine of patients suffering from myocardial infarction (9–11), whereas psoriasis-associated FABP (E-FABP) was among a number of marker proteins detected in the urine of bladder cancer patients (11). E-FABP, also called a melanogenic inhibitor, was shown to reduce cell proliferation in melanoma cells when added exogenously, whereas normal skin fibroblasts were unaffected (36). E-FABP and A-FABP have been associated with more differentiated phenotypes of cells. Our findings show a unique pattern of expression, and we observed a significant loss of A-FABP expression in prostate tumor cells when compared with the normal primary cells. The normal cells showed high levels of A-FABP and E-FABP. The expression of E-FABP was also 6–50-fold lower in cancer cells when compared with the normal cells. In human prostate tissue samples, we observed a dramatic reduction ( $\sim 10$ -fold) of the levels of E-FABP in tumor samples when compared with the normal tissue samples. Similar loss of A-FABP has been reported with progression of human bladder transitional cell carcinoma (18, 37). The presence of A-FABP correlated with the grade and stage of the disease. The A-FABP protein was present in high levels in grades I and II transitional

cell carcinomas, whereas grade III had a 37% reduction and grade IV had no A-FABP expression (37). A-FABP may act as a growth inhibitor similar to MDGI (now identified as being homologous with H-FABP) protein in breast cancer. Loss of A-FABP expression was proposed to be a prognostic marker for aggressive bladder cancer. In prostate cancer, also it seems that the loss of A-FABP correlates directly with tumor cell status. The human breast cancer cell line MCF7 does not express heart-type fatty acid-binding protein, a marker protein for differentiated mammary gland. MCF7 cells transfected with the bovine H-FABP cDNA expressed the corresponding protein and were characterized by growth inhibition and lower tumorigenicity in nude mice (17, 38). The transfection of H-FABP into these cells was able to alter the uptake of fatty acids. Compared with control cells, the uptake of radioactively labeled palmitic acid and oleic acid was increased by 67% in H-FABP-expressing transfectants, demonstrating a stimulatory role for this FABP-type in fatty acid metabolism (38).

The novel finding in the current study is that in addition to the decreased heart-type FABPs in the prostate cancer cells, there was a dramatic increase in I- and L-FABPs. L-FABP has been shown previously to be elevated significantly in metastatic or regenerating liver *versus* normal liver (13, 15). 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, especially in proportion to A- or E-FABP, can suggest over-expression of this protein, which in turn may indicate the presence of cancer in prostate cells.

Wolfrum *et al.* (39) showed a direct correlation between the content of L-FABP and fatty acid uptake in human hepatoma HepG2 cells. Potent carcinogens and peroxisome proliferators bezafibrate and Wy14, 643 were able to double the expression of L-FABP levels and also the uptake of fatty acids. In that study, the use of antisense ODN complementary to L-FABP decreased fatty acid uptake in these transfectants (39). Thus

levels of these FABP may regulate fatty acid uptake in other cell systems as well.

Recently Jing *et al.* (40), using differential display, identified a mRNA for human cutaneous fatty acid-binding protein (C-FABP) as a metastasis inducer in breast and prostate carcinoma cell lines. They suggest that C-FABP, which is a homologue of E-FABP, can induce metastasis. This is in contrast to what we have shown in the present study, where there is down-regulation of E-FABP in cancer cells when compared with the normal cells. However that study also found the level of E-FABP to be very low in primary tumors (40). It is possible that E-FABP expression increases during metastasis, and that possibility will require additional investigation. There are multiple reports on the changes in the levels of these FABPs, in a variety of cell systems, to correlate with the normal or tumor stage.

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 an increase in the 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 only in well-differentiated prostate cancer cells and biopsy samples. A-FABP and B-FABP 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.

We have shown that, in prostate cancer cells, by overexpressing A-FABP we could inhibit cell proliferation and induce apoptosis. When DU 145 cells were transfected with A-FABP, we observed an increase in apoptotic cells, suggesting that these FABPs are crucial in cell proliferation.<sup>4</sup> This finding suggests that there is a direct effect on cell function from changing the expression of these FABP proteins. Using a different approach, we found that by blocking the expression of L-FABP (using antisense ODN for L-FABP) there was a corresponding block in the proliferation of DU 145 cells along with the induction of apoptosis in a concentration-dependent manner. This observation relates to more than just low levels any one FABP. For example, a cell line constitutively expressing low levels of L-FABP, such as PC-3, shows high levels of I-FABP, a FABP that also seems to be a positive trigger for proliferation. It is not just absolute levels of any one FABP, but rather the overall balance of the repertoire of FABPs that appear to regulate cellular functions. Also, by using antisense to E-FABP in these cells, we induced an increase in cell proliferation. These results suggest that E- and A-FABP may act as a tumor suppressor in prostate cells similar to MDGI (H-FABP) in MCF-7 cells (16, 17). In essence, low levels of L-, I-, and B- or high levels of A- and E-FABPs inhibited tumor cell proliferation. Their characteristics of binding to lipids and to hormones could each contribute to the role as regulatory molecules. We have shown that upon altering the patterns of FABPs in cells, we can influence cellular survival. This has the potential to be an important step toward designing therapeutic strategies for prostate cancer.

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). Either by increasing the heart-type FABPs (by transfection) or decreasing the liver-type FABPs (by antisense ODN), one can control the balance of apoptosis and proliferation in prostate cancer cells. We suggest that there is a distinct balance between the levels of various FABPs in a cell which might predict the stage and degree of malignancy. A- and B-FABP and myelin-FABP may be key and support the notion that level(s) of FABP(s) will correlate with the 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. The mechanism of action of these FABPs in normal and cancer cells are currently under investigation, which will provide a better understanding of the role of bioactive lipids in prostate cancer.

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# Clinical Cancer Research

## Expression Pattern of Fatty Acid-binding Proteins in Human Normal and Cancer Prostate Cells and Tissues

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