Identification of the Messenger RNA for Human Cutaneous Fatty Acid-binding Protein as a Metastasis Inducer

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ABSTRACT

Using our recently developed systematic differential display and complete comparison of gene expression approaches combined with other methods, we have identified a large number of mRNAs that are expressed differentially between benign and malignant human cells. One such mRNA that is common to prostate and breast carcinoma cell lines encodes the human cutaneous fatty acid-binding protein (C-FABP). Northern and slot blot analyses confirm that the expression levels of C-FABP mRNA in the malignant prostate and breast carcinoma cell lines are 4.9 ± 0.9- to 16.9 ± 2.1-fold higher than those expressed in the benign cell lines. A similar difference between the benign and malignant cell lines was also detected at the protein level. In situ hybridization experiments have detected overexpression of the mRNA for C-FABP in human prostate carcinoma tissues. Transfection of a C-FABP expression construct into the benign, nonmetastatic rat mammary epithelial cell line Rama 37 and inoculation of the C-FABP expression transfectants into syngeneic Wistar-Furth rats produce a significant number (P < 0.05) of animals with metastases (6 of 26 animals), whereas the control transfectants generated by the vector alone yield no such metastases. Measurements of mRNA and protein levels with Northern and Western blotting show that C-FABP is not expressed in the control transfectant cells produced by the vector alone but is highly expressed in the pool of C-FABP transfectants and the sublines established from their metastases. Immunochemical staining with antibodies to C-FABP shows that C-FABP is not expressed in the primary tumors developed from the control transfectants that have failed to metastasize, but it is expressed in both the primary tumors developed from the C-FABP transfectants and their metastases. Re inoculation of the sublines established from metastases in syngeneic rats has produced a higher proportion (50%) of animals (7 of 14 animals) with metastases than that obtained in the first-round inoculations, indicating that the metastatic clones have been preferentially selected from the original pool of metastatic and nonmetastatic transfectant clones. These results have demonstrated that elevated expression of C-FABP can induce metastasis and that metastatic capability has been transferred in a genetically dominated manner in this Rama 37 model. Thus, we suggest that C-FABP is a metastasis-inducing gene, and under suitable conditions, it may induce metastasis of some human cancers.

INTRODUCTION

Metastasis, the malignant dissemination of cancer cells from their primary sites of origin to distant secondary sites in the body, is the major cause of death from cancer. A number of largely sequential genetic changes are thought to be necessary before a tumor cell is capable of metastasizing (1). These changes include the decreased activities of the metastasis suppressor genes, e.g., Kitl (2), and the increased activities of the metastasis promoter genes, e.g., p9Ka (3).

It is likely that some of the highly expressed genes in malignant metastatic cells are candidates for metastasis-promoting genes, whereas some of the highly expressed genes in the benign nonmetastatic cells are candidates for metastasis-suppressing genes (4). Analysis of differentially expressed genes between nonmetastatic and metastatic cells can provide valuable data for further investigations leading to the identification of important genes involved in the process of cancer metastasis.

The early technique used for analysis of differential gene expression was subtractive hybridization, and this technique was followed by that of differential display or RNA fingerprinting. Because of its advantages over subtractive hybridization and its simplicity for individual bench operation, differential display has now become a widely used method. Despite its popularity, the original technique suffered from the fact that only differences in relatively abundant mRNAs could be detected, and many of those apparent differences were later found to be artifacts. Recently, we have developed two novel strategies named SDD (5) and CCGE (6). Whereas SDD is used to analyze entire differentially expressed genes from the 3’ end of cDNAs, CCGE is used to analyze the cDNA fragments within or near to the protein-coding region.

To identify and isolate the possible genes involved in malignant dissemination of prostate and breast cancer cells, we have adopted a strategy consisting of several rounds of “subtractive selections” of the candidate genes. By excluding genes that do not fulfill each selection criteria, the genes involved in metastasis would eventually be identified. In the first round of selection, we used SDD and CCGE to display the entire cDNA species in a benign cell line and in a malignant cell line and identified a number of differentially expressed genes. In the second round of selection, we used the cDNA fragments obtained from the first round of selection as probes to screen a wider range of benign and malignant cell lines by Northern and slot blot analyses and greatly reduced the number of candidates. In the third round of selection, in situ hybridization was used to detect the expression of the candidate mRNAs in human prostate tissues. This step has further excluded some mRNAs from the remaining candidates. In the last round of selection, we transfected the full-length cDNAs of the remaining candidates into appropriate cell models that are suitable for assaying either the metastasis-promoting or metastasis-suppressing activities of a gene. Whether or not the candidate genes are involved in metastasis is eventually determined by their ability to promote or suppress the malignant dissemination of the DNA recipient cells in vivo.

In the work described in this report, we have used the SDD approach combined with Northern and Western blotting, and we have characterized one cDNA fragment by DNA sequencing as encoding part of the gene corresponding to human C-FABP, which is also called

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3 The abbreviations used are: SDD, systematic differential display; CCGE, complete comparison of gene expression; C-FABP, cutaneous fatty acid-binding protein; PA-FABP, psoriasis-associated fatty acid-binding protein; RT-PCR, reverse transcription-PCR; GAPP, glyceraldehyde 3-phosphate dehydrogenase; ECL, enhanced chemiluminescence; BPH, benign prostate hyperplasia; VEGF, vascular endothelial growth factor; PPAR, peroxisome proliferator-activated receptor.
epidermal fatty acid-binding protein or PA-FABP and was previously found to be abundantly expressed in the psoriatic skin (7). Detection of C-FABP mRNA by in situ hybridization showed that this gene was overexpressed in prostate carcinoma tissues. To investigate the possible role of the overexpressed C-FABP gene in promoting metastasis, we transfected full-length C-FABP cDNA into the benign, nonmetastatic Rama 37 model cell line (8) and subsequently tested the metastatic capability of the resultant transfectants in syngeneic Wistar-Furth rats. The C-FABP gene induced the benign cells to disseminate to secondary sites when it was overexpressed in the recipient cells in this system.

MATERIALS AND METHODS

Cell Lines. Four human prostate cell lines were used in this work: (a) the benign PNT-2 cell line established from human prostate tissue (9–11); (b) the weakly malignant LNCaP cell line established from prostate carcinoma (12); and (c) the two highly malignant cell lines PC-3 (13) and Du-145 (14) established from a rib metastasis and a brain metastasis of prostate carcinoma, respectively. The two human breast cell lines used in this work are the benign cell line Huma 121 established previously in our laboratory (15) and the widely used malignant cell line MCF-7 (16). Although these human cell lines have been well characterized previously, in this work, their benign and malignant properties have been further confirmed by examining their ability to invade a basement membrane matrix (Table 1) using an in vitro invasion assay (17). The DNA recipient cell line used in the DNA transfection assay was the benign, nonmetastatic rat model cell line Rama 37 (8). The rat metastatic sublines Met-1 and Met-2 were established from an auxiliary lymph node and a lung metastasis, respectively, using primary tissue culture procedures similar to those described previously (18). These metastases were established through the transfection of benign Rama 37 cells with the C-FABP gene. The human and rat cell lines were grown as monolayer cultures in RPMI 1640 (human cells) and DMEM (rat cells) supplemented with 10% (v/v) FCS, hydrocortisone (5 μg/ml), and insulin (5 μg/ml; mammary cells).

Isolation of DNA, RNA, and Protein. For preparation of DNA, RNA, and protein, cells from each cell line were grown to about 80% confluence in Petri dishes (13.5 cm in diameter; Life Technologies, Inc., Paisley, United Kingdom), washed with sodium PBS (pH 7.4), lysed by a 4 M guanidine isothiocyanate solution, and layered onto 5 ml of CsCl in 14-ml Sorvall (Wilmington, DE) ultracentrifuge tubes. The genomic DNA, total cellular RNA, and total cellular protein were separated by gradient centrifugation (19). The polyadenylated RNA was isolated from the total RNA with a Oligotex mRNA mini-kit (Qiagen GmbH and Qiagen Inc., Hilden, Germany), and its integrity was verified by the presence of an undegraded band following Northern hybridization to a radioactively labeled actin probe.

Table 1 Relative levels of C-FABP mRNA and protein in different human prostate and mammary cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>Percentage of invading cells</th>
<th>Relative levels of C-FABP expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNT-2</td>
<td>Benign prostate</td>
<td>0.0</td>
<td>1.1</td>
</tr>
<tr>
<td>LNCaP</td>
<td>Prostatic carcinoma</td>
<td>1.1</td>
<td>4.9 ± 0.9</td>
</tr>
<tr>
<td>PC-3</td>
<td>Rib metastasis of prostatic carcinoma</td>
<td>10.0</td>
<td>16.9 ± 2.1</td>
</tr>
<tr>
<td>Du-145</td>
<td>Brain metastasis of prostatic carcinoma</td>
<td>5.2</td>
<td>14.8 ± 2.1</td>
</tr>
<tr>
<td>Huma 121</td>
<td>Breast</td>
<td>0.0</td>
<td>1.0</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Breast</td>
<td>1.8</td>
<td>6.1 ± 1.1</td>
</tr>
</tbody>
</table>

References describing these cell lines are cited in the text.

All cell lines were previously well characterized. To further confirm their benign and malignant properties, an in vitro invasion assay (17) was used to examine the ability of the cells to invade a basement membrane matrix. The invasion ability of each cell line was expressed by the percentage of the cells invading the basement membrane matrix.

Quantitative analyses of mRNA levels of C-FABP were performed by slot blotting. Samples of total RNA from different human cell lines were divided into a prostate group and a breast group for comparison. Increasing amounts of mRNA (0.1–4.8 μg) from each cell line were loaded onto duplicate membranes. One membrane was used for hybridization with the C-FABP cDNA probe; and the other was used for hybridization with GAPD cDNA. The levels of mRNA in different cell lines within each group were compared, and the mRNA levels in the benign prostate (PNT-2) and breast (Huma 121) cell lines were set at 1. The mean relative levels and SDs were obtained from three separate experiments. The levels of C-FABP protein in malignant cell lines of both prostate and breast sources were significantly higher than those in the corresponding benign cell lines (Student’s t-test, P < 0.01).

SDD. SDD was performed to compare the prostate and breast cells to identify genes that were differentially expressed in these two cell types. The procedures for performing SDD analysis were the same as those described previously (5). The anchor primer used for first-strand cDNA transcription was 5′-CTGCTGGTGT+15CA-3′. The primer pair used in this work was one of the 192 possible combinations that amplified the cDNA subset, which was later found to contain the representative cDNA fragment for C-FABP. The positive primer sequence at the nonbiotinylated end was 5′-ACAAGCCACGCCCGCATAAA-3′. Similarly, the negative strand primer at the biotinylated end was 5′-CTGCTGGTGT+15AG-3′. The nucleotide sequences of the recovered fragments originating from the prostate and breast cell lines were determined twice using an automated sequencer (ABI 377; Applied Biosystems). The cDNA fragments were identified as part of human C-FABP cDNA by comparing sequence homology through computer searches of the EMBL data Library with BLAST.

Construction of the C-FABP Expression Vector. Full-length C-FABP cDNA was obtained by a RT-PCR procedure similar to that described previously (5). After confirming that there was no formation of internal duplexes and hairpins and no homology with other sequences in the EMBL data Library, the first 22 bases of C-FABP cDNA from the 5′ end (5′-ACCGCGGACCGACGACCCCTC-3′) were used as a positive strand primer for RT-PCR. One μg of total RNA from the malignant prostate cell line PC-3 was transcribed into first-strand cDNA with a hybrid primer containing a XbaI recognition sequence, a poly(T) region, and two more bases at its 3′ end (5′-GGTCTAGATTG-3′). This hybrid primer was also used as the negative strand primer for RT-PCR. Full-length C-FABP cDNA amplified by RT-PCR was blunt-ended and inserted into a PCR-Blunt vector using the Zero Blunt PCR Cloning Kit (Invitrogen). C-FABP was then excised from the PCR-Blunt vector by EcoRI and XhoI and inserted into the pSVneo plasmid, which had been linearized by digestion with EcoRI and XhoI. The correct cloning of C-FABP cDNA into the pSVneo vector was confirmed by automated nucleotide sequence analysis through the junctions.

Transfection of DNA and the In Vivo Assay for Metastasis. Exponentially growing benign Rama 37 cells were harvested, seeded at a density of 0.5–0.75 × 10⁶ cells/10 ml DMEM in each 10-cm-diameter Petri dish and transfected with 20 μg of pSV-C-FABP expression vector or pSVneo vector alone. The method for DNA transfection was the same as that described previously (20). The resultant cells were passaged at a 1:10 dilution in a selective medium containing 1 mg/ml Geneticin (G418), which was replaced every 3–4 days thereafter. After a week or so, when cell colonies had grown to more than 1.5–3.5 mm in diameter, the colonies of cells were pooled and expanded by growth in culture to yield pSV-C-FABP-R37 transfectants or control pSV-R37 transfectants.

The pSV-C-FABP-R37 transfectants and the control pSV-R37 cells were injected at a density of 2 × 10⁶ cells/0.2 ml PBS into animals in the control and experimental groups (each group consisted 30 animals; 4–6-week-old female Wistar-Furth rats; Wistar OLA strain) at a single s.c. site in the right inguinal

2391
mammary fat pad. Those animals that developed ulcerations in their primary tumors were killed immediately and excluded from further study. All surviving animals were autopsied 3 months after the injection, and the lungs, liver, spleen, kidney, heart, and axillary lymph nodes were examined for gross metastases. Samples of primary tumors and tissues taken at autopsy were fixed in Methacarn (methanol:inhibibiose:acetic acid, 6:3:1) or neutral buffered formal saline, processed conventionally, and embedded in paraffin wax. Sections of each tissue were examined for metastases by at least two independent observers. To confirm the mammary epithelial origin and primary site of the metastases, immunocytochemical staining of the tissue sections with antibodies to human calius keratin, rat milk fat globular membrane, and vimentin was performed using a procedure similar to that described previously (3, 21). The sections were counterstained with Gill’s hematoxylin.

Detection of DNA, RNA, and Protein. Southern blot hybridization for detection of the transfected genes was performed according to standard procedures. The drug resistance neo gene probe was an 881-bp fragment obtained by digesting the pSVneo vector with HindIII and BssHII. The C-FABP probe was a 452-bp fragment recovered from the SDD denaturing gel. The cDNA probes were radioactively labeled with [α-32P]dCTP to a specific activity of 0.6–6 × 10^6 dpm/μg DNA using a random-primed labeling kit (Boehringer Mannheim Biochemica). The radioactively labeled probes were incubated separately with the immobilized DNA fragments on the nylon membranes in a hybridization oven (Techne HB-10; Philip Harris, Manchester, United Kingdom) at 42°C for 16 h, using predetermined optimum hybridization conditions. The radioactive images of the bound probes were detected by autoradiography.

For Northern blotting to detect mRNA, total RNA samples (10 μg each) prepared from the cell lines were electrophoresed under denaturing conditions with formaldehyde in a 0.8% (w/v) agarose gel. After washing the gel, the separated RNAs were transferred to nylon membranes (Hybond; Amersham, United Kingdom) and cross-linked by a brief exposure to a 302 nm UV light. The membranes were incubated at 42°C for 4 h in a prehybridization buffer and then hybridized with the radioactively labeled C-FABP probe for 16 h at 42°C under the conditions described previously (22). Radioactivity bound to the washed membrane was detected by autoradiography against Kodak XAR films.

Slot blotting was performed to quantify the levels of C-FABP mRNA in different cell lines. Increasing amounts of mRNA, from 0.1–4.8 μg, were loaded onto nylon membranes (Hybond; Amersham) using a slot blot apparatus (Bio-Rad, Hercules, CA) coupled with a brief exposure to UV light. For each experiment, duplicated membranes were hybridized with the radioactively labeled C-FABP probe and a control GAPD probe. The bound radioactivity was detected by autoradiography, and autoradiographic images were scanned with an IS-1000 digital image system (Alpha Innotech, San Leandro, CA). Relative mRNA levels were quantified by measuring the peak areas of transmitted light. The best-fit straight lines over the linear parts of plots of peak areas against the amount of RNA/slot were calculated by linear regression. Possible loading artifacts were normalized with the constitutively expressed GAPD mRNA.

The C-FABP mRNA in human prostate tissue was detected by in situ hybridization. The 452-bp C-FABP cDNA fragment obtained from SDD was inserted in a pTF73 plasmid through a blunt-end ligation, and the C-FABP probe (the antisense strand) was transcribed with T7 RNA polymerases, using a method similar to that described previously (22). The digoxigenin label was incorporated into the single-stranded probe by using a probe labeling kit, following the manufacturer’s instructions (Boehringer Mannheim Biochemica). The in situ hybridization was performed under RNase- and DNase-free conditions using a nonradioactive in situ hybridization kit (Boehringer Mannheim Biochemica). The sections were counterstained with methyl green. The sections were examined for metastases by at least two independent observers. To confirm the mammary epithelial origin and primary site of the metastases, the sections were counterstained with Gill’s hematoxylin.

RESULTS

Identification and Verification of C-FABP as a Differentially Expressed Gene. The recently developed SDD approach has been used to perform a systematic assessment of profiles of differential human gene expression between benign prostatic cell line PNT-2 and malignant cell line LNCaP and between the benign breast cell line Huma 121 derived from fibrocytic tissue and the malignant cell line MCF-7. One subset of cDNAs, which was selected from 192 subsets from each pair of cell lines, contained a C-FABP cDNA fragment. This subset from both pairs of cell lines was amplified by the same primer pair and displayed in denaturing polyacrylamide gels (Fig. 1). In these two displayed subsets of cDNA fragments, several bands exhibited different levels of intensity between the benign and malignant cell lines. The arrow points to a highly expressed band in the malignant LNCaP (Fig. 1A) and MCF-7 cells (Fig. 1B), but this band was weak in the benign PNT-2 and Huma 121.

Fig. 1. Detection of differential expression of the C-FABP gene between benign and malignant human cells. The SDD approach was used to identify the profiles of differentially expressed mRNAs extracted from different cell lines. The expression profile of a subset of cDNAs of a benign cell line and a malignant cell line originating from human prostate (A) and a benign cell line and a malignant cell line originating from human breast (B) is shown. Molecular size markers are shown as the number of bp. The C-FABP expression pattern on all four lanes in both panels was produced by the same primer pair used in the SDD-PCR. Lane 1, the benign human prostatic cell line PNT-2. Lane 2, the malignant human prostatic cell line LNCaP. Lane 3, the benign human mammary cell line Huma 121. Lane 4, the malignant human mammary cell line MCF-7. The arrowhead points to the position of the band containing the C-FABP cDNA fragment.
is barely detectable in the benign PNT-2 and Huma 121 cells. Nucleotide sequence analysis of the corresponding cDNA fragments recovered from the denaturing gels revealed that they were identical and corresponded to a 452-bp fragment (excluding the primer sequence at both ends) that showed 100% homology to the cDNA coding for human C-FABP (7).

To confirm the differential expression patterns between the benign and the malignant cells repeatedly obtained by SDD, Northern and Western blots were performed to detect the C-FABP at both mRNA and protein levels in four human prostate cell lines and two human breast cell lines (Fig. 2). Similar to the result obtained with SDD, the level of C-FABP mRNA in the benign prostate PNT-2 cells was not detectable by Northern blotting, whereas a band of the same size as C-FABP mRNA (662 bp) was detected in all three malignant prostate cell lines, albeit at different levels (Fig. 2A). Northern blotting also detected a pattern of differential expression of C-FABP mRNA very similar to that detected with SDD between the benign Huma 121 and the malignant MCF-7 breast cells (Fig. 2A). Western blotting detected a single Mr 15,000 C-FABP protein band in all three malignant prostate cell lines (LNCaP, PC-3, and Du-145) and the malignant breast cell line MCF-7 (Fig. 2B). The expression of C-FABP protein in the benign breast cell line Huma 121 was much lower than that in the corresponding malignant cell line MCF-7, whereas in benign prostate cell lines, its level was barely detectable.

The origins, invasiveness, and the mRNA and protein levels of C-FABP in these cell lines are shown in Table 1. When the cell lines were subjected to the in vitro assay for invasiveness, no benign prostate cell line PNT-2 or benign breast cell line Huma 121 cells were able to invade the basement membrane matrix. For the two malignant cell lines, LNCaP (prostate) and MCF-7 (breast), only 1.1% and 1.8% of the cells, respectively, invaded the basement membrane matrix. The highest level of invasiveness was detected in the malignant metastasis-derived prostate cell line PC-3, with 10% of the cells invading the basement membrane matrix, and this was followed by the malignant metastasis-derived Du-145 cell line, with 5.2% of the cells invading the basement membrane matrix. Quantification of the C-FABP mRNA and protein levels in the different cell lines by slot blot and Western blotting showed similar trends to those detected by Northern blot hybridization, and the level of C-FABP in these cell lines appeared to increase with increasing invasive ability. Thus, among the four prostatic cell lines, the level of C-FABP mRNA in the malignant cell line LNCaP was nearly five times that found in the benign PNT-2 cells, and this level was increased further by nearly 17- and 15-fold, respectively, in the malignant metastasis-derived PC-3 and Du-145 cell lines. In the human breast cell lines, the level of C-FABP mRNA in the malignant MCF-7 cells was more than 6-fold higher than that expressed in the benign Huma 121 cells. At the protein level, C-FABP was also increased in malignant cells when compared with that in the benign cells. For the prostatic cell lines, the level of C-FABP in the malignant cell line LNCaP was 3.9 times that expressed in the benign PNT-2 cell line. The malignant metastasis-derived cell lines PC-3 and Du-145 expressed 15.2 and 11.3 times, respectively, as much C-FABP as the benign PNT-2 cells. For the breast cell lines, the protein level of C-FABP in the malignant MCF-7 cells was 4.1 times that detected in the benign Huma 121 cells (Table 1).

Expression of the mRNA for C-FABP in Human Prostate Tissues. A C-FABP riboprobe was used to hybridize the mRNA in situ from 130 samples (66 benign and 64 malignant samples) of prostate tissues. This probe detected very different patterns of expression of C-FABP mRNA between the BPHs and the malignant carcinomas (Table 2). Fifty-nine of the 66 (89.4%) BPH samples were classified as negative (<10% of the epithelial cells were stained) by in situ hybridization, whereas the seven of them (10.6%) were classified as partially positive (10–75% of the epithelial cells were stained). The number of malignant tissue samples expressing C-FABP mRNA was significantly higher than the number of BPHs expressing C-FABP mRNA (Fisher’s exact test, P < 0.01), although no significant difference was seen between the carcinomas with lower Gleason scores (scores of 1–5) and those with higher Gleason scores (scores of 5–10). Among the 39 samples with Gleason scores of 1–5, 21.5% of the benign prostate tissue was detected by in situ hybridization. The result was assessed by examining 5–10 fields in two sections from each tissue sample. For each field, approximately 100 cells were examined. The samples with less than 10% of cells stained were classified as negative, those with 10–75% of cells stained were classified as partially positive, and samples with more than 75% of cells expressing C-FABP mRNA were classified as positive. The prostate carcinomas were graded by their combined Gleason scores (23). The C-FABP expression in the carcinoma tissues is significantly higher than that in the BPHs, but the differences between the carcinomas with scores of 1–5 and those with scores of 6–10 are not significant (Fisher’s exact test, P > 0.01).

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Total no.</th>
<th>Negative</th>
<th>Partially Positive</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPHs</td>
<td>66</td>
<td>59</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Carcinomas with Gleason (scores 1–5)</td>
<td>39</td>
<td>8</td>
<td>21.5</td>
<td>4</td>
</tr>
<tr>
<td>Carcinomas with Gleason (scores 6–10)</td>
<td>25</td>
<td>5</td>
<td>20</td>
<td>3</td>
</tr>
</tbody>
</table>

* The C-FABP mRNA in human prostate tissue was detected by in situ hybridization. The result was assessed by examining 5–10 fields in two sections from each tissue sample. For each field, approximately 100 cells were examined. The samples with less than 10% of cells stained were classified as negative, those with 10–75% of cells stained were classified as partially positive, and samples with more than 75% of cells expressing C-FABP mRNA were classified as positive. The prostate carcinomas were graded by their combined Gleason scores (23). The C-FABP expression in the carcinoma tissues is significantly higher than that in the BPHs, but the differences between the carcinomas with scores of 1–5 and those with scores of 6–10 are not significant (Fisher’s exact test, P > 0.01).
cDNAs were inserted into the mammalian expression vector pSV neo, and the construct was transfected into the Rama 37 cell line. In a control, the pSV neo vector alone, without the insert, was also transfected into the Rama 37 cells. When both sets of transfectant cells were cultured in a selection medium containing Geneticin, small cell colonies became visible in about 10 days with similar transfection efficiencies \((1.99 \times 10^{-5} \text{ and } 2.07 \times 10^{-5})\) for the inserted vector and the vector alone, respectively. The colonies from each transfection experiment were combined to form two separate pools of transfectant cells; those containing the C-FABP expression construct were termed pSV-C-FABP-R37, and those containing only the pSV neo vector were termed pSV-R37. No significant differences were observed in the growth rates between the two transfectant cell pools or between the transfectant cell pools and the construct was transfected into the Rama 37 cells showed a distinct epithelial morphology with some partially differentiated invasive carcinomas (large arrow) and in single cell invasion to the stroma (small arrow). Magnification, \(\times 120\). Bar in a, 150 \(\mu m\).

DNA Transfection and the Metastasis Assay. The C-FABP cDNA was inserted into the mammalian expression vector pSV neo, and the construct was transfected into the benign rat mammary epithelial cell line Rama 37. In a control, the pSV neo vector alone, without the insert, was also transfected into the Rama 37 cells. When both sets of transfectant cells were cultured in a selection medium containing Geneticin, small cell colonies became visible in about 10 days with similar transfection efficiencies \((1.99 \times 10^{-5} \text{ and } 2.07 \times 10^{-5})\) for the inserted vector and the vector alone, respectively. The colonies from each transfection experiment were combined to form two separate pools of transfectant cells; those containing the C-FABP expression construct were termed pSV-C-FABP-R37, and those containing only the pSV neo vector were termed pSV-R37. No significant differences were observed in the growth rates between the two transfectant cell pools or between the transfectant cell pools and their parental Rama 37 cells (data not shown).

The pooled transfectants were tested for their biological activity in syngeneic rats. Two groups of 30 Wistar-Furth (OLA strain) female 4–6-week-old rats were injected with either pSV-C-FABP-R37 cells or the control pSV-R37 cells. Four animals from the experimental group that developed ulcerated primary tumors and had to be killed prematurely were excluded from further study. The remaining 56 animals (26 animals in the experimental group and 30 animals in the control group) developed primary tumors (100%). The mean latent periods before the appearance of primary tumors in both control and experimental groups were not significantly different (Student’s \(t\) test, \(P > 0.05\)), with means of 14.3 and 14 days, respectively. At autopsy, the sizes of primary tumors in each group of animals varied from 1–4 cm in diameter. The tumors consisted predominantly of spindle cells, with some glandular elements. Among the remaining 26 animals inoculated with pSV-C-FABP-R37 cells, a significant number of rats (6 of 26 animals; 23.1%) developed metastases (Fisher’s exact test, \(P < 0.02\)). The histological appearance of the metastases was similar to that of the corresponding primary tumors. One of the metastases, which developed in the right axillary lymph node of a rat, was so large (1 cm in diameter) that most of the lymphatic tissue was replaced with tumor cells (Fig. 7c). A visible pulmonary metastasis (5 mm in diameter) was found in another rat during autopsy. The remaining four lung metastases in four different rats were multiple micrometastases with a small “cannon ball” morphology (Fig. 7d) found on microscopic examination. The mammary epithelial origin of the metastases was confirmed by their immunocytochemical staining with antibodies to markers of epithelial cells including antibodies to human callus keratin and to rat milk fat globular membrane (21); all six metastases stained positively (data not shown). Antibodies to vimentin also stained these metastases and their primary tumors (data not shown), confirming their origin from individual cells of the primary tumors. No metastases were identified at autopsy or on subsequent histological examinations in all 30 animals inoculated with control pSV-R37 cells.

Establishment of Metastatic Variants and Detection of the Transfected DNA. A small fraction of the lymph node metastasis and part of the visible pulmonary metastasis were collected at autopsy for primary culture in DMEM containing Geneticin. Two cell lines, Met-1 (from the lymph node metastasis) and Met-2 (from the lung metastasis), were successfully reestablished. Whereas the parental Rama 37 cells showed a distinct epithelial morphology with some elongated cells, the C-FABP transfectants pSV-C-FABP-R37 were predominantly spindle-shaped cells. The Met-1 and Met-2 cells were spindle-shaped, fusiform in appearance, and poorly cohesive (Fig. 4). When reimplanted into seven syngeneic Wistar-Furth rats, both Met-1 and Met-2 produced primary tumors (Table 3) in all of the rats (100%). In addition, Met-1 cells produced multiple lung metastases in three different rats (43%), and Met-2 cells produced similar multiple lung metastases in four different rats (57%). The average rate of

![Fig. 3](image1.png) Detection of the mRNA for C-FABP in human prostate tissues by in situ hybridization with an antisense riboprobe. The purple color indicates the expression of C-FABP mRNA. Sections were counterstained with methyl green. a, an example showing negative staining, which was observed in most BPHs; b, an example of partial staining, observed in some BPH samples; the large arrow points to some epithelial cells expressing C-FABP, and the small arrows point to cells that do not express C-FABP. c, positive staining was observed in moderately differentiated carcinomas. d, strong positive staining was observed in poorly differentiated invasive carcinomas (large arrow) and in a single cell invasion to the stroma (small arrow). Magnification, \(\times 120\). Bar in a, 150 \(\mu m\).

![Fig. 4](image2.png) Morphological appearance of the benign Rama 37 cells, C-FABP transfectants, and their two metastatic sublines. a, parental Rama 37 cultures are predominantly cuboidal epithelial cells in morphology, with a small number of more elongated cells. b, C-FABP transfectants showing predominately spindle-shaped cells in a cross-cross morphology, with a small number of cuboidal epithelial cells. c, Met-1 cells are a metastatic variant reestablished from a lymphatic metastasis produced by inoculating a rat with pSV-C-FABP-R37 cells. d, Met-2 cells are a metastatic variant reestablished from a pulmonary metastasis produced by inoculating a separate rat with pSV-PA-FABP-R37 cells. Both metastatic variants have a loosely adherent, spindle-shaped, cross-cross morphology. Bar, 50 \(\mu m\); magnification, \(\times 200\).
metastasis (50%) produced in the animals (7 of 14 animals) inoculated with Met-1 and Met-2 was significantly higher than that observed in the first round of inoculation with the C-FABP-transfectants (Fisher’s exact test, \( P < 0.03 \)). The lung nodules varied in sizes from tiny nodules to a metastasis with a size of 3 mm in diameter. All seven metastases were identified in the lungs of the animals during the autopsy.

To confirm that the Met-1 and Met-2 cells reestablished from the metastases were related to the parental pSV-C-FABP-R37 cells, they were analyzed by Southern blotting for the neo and human C-FABP genes. When probed with a neo CDNA fragment, no hybridization was detected with the DNA extracted from the parental Rama 37 cells (Fig. 5A, Lane 1). However, the neo gene was detected in DNA samples extracted from the pSV-R37 control cells (Fig. 5A, Lane 2), the pSV-C-FABP-R37 transfectant cells (Fig. 5A, Lane 3), and the metastasis-derived Met-1 (Fig. 5A, Lane 4) and Met-2 cells (Fig. 5A, Lane 5). The sizes of major neo hybridizing bands in the pSV-C-FABP-R37 cells were between 3 and 20 kbp, with a complex pattern. Much fewer bands with simpler patterns were detected in the metastasis-derived Met-1 and Met-2 cells. When the DNA samples were incubated with the C-FABP cDNA probe, no hybridization band was detected in the control cells containing only pSV vector DNA (Fig. 5B, Lane 1). However, the C-FABP gene was detected in the DNA samples extracted from the transfectant pSV-C-FABP-R37 cells (Fig. 5B, Lane 2), and the metastasis-derived Met-1 (Fig. 5B, Lane 3) and Met-2 cells (Fig. 5B, Lane 4). There were more bands and stronger bands in Met-1 than in Met-2 cells, but both probably represented subsets of the more complex pattern in the pooled pSV-C-FABP-R37 transfectants, which was similar to that found for hybridization to the neo gene.

**Detection of C-FABP Expression in Rat Cells.** No C-FABP mRNA was detected in the control transfectant pSV-R37 cells, but it was highly expressed in the transfectant pSV-C-FABP-R37 cells and in metastatic sublines Met-1 and Met-2 cells (Fig. 6A). A similar expression pattern was observed at the protein level (Fig. 6B). Quantitative analyses of C-FABP showed that neither mRNA nor protein was detected in the parental Rama 37 cells or in the control transfectant pSV-R37 cells. However, C-FABP was highly expressed in the transfectant pSV-C-FABP-R37 cells and in the metastatic Met-1 and Met-2 cells. The expression of C-FGBP in the transfectant pSV-C-FABP-R37 cells was 1.3 and 1.1 times higher at the mRNA and the protein levels, respectively, than that in the metastatic Met-1 cells. The expression of C-FGBP in the metastatic Met-2 cells was 0.84 and 0.72 times that in the Met-1 cells at the mRNA and protein levels, respectively (Table 3), but the differences in C-FGBP expression (at the mRNA and protein levels, respectively) among the Met-1, Met-2, and pSV-C-FABP-R37 cells were not significant (Student’s t test, \( P > 0.05 \)).

The presence of C-FGBP in the primary tumors and metastases was detected by immunocytochemical staining with antibodies to C-FGBP (Fig. 7). No staining was observed in the primary tumors produced by the control pSV-R37 cells (Fig. 7A). Strong staining was observed in the primary tumors (Fig. 7B) produced by the pooled transfectant pSV-PA-FABP-R37 cells, in the lymph node metastasis, and in all five pulmonary metastases with the antibody to C-FGBP (Fig. 7C and D).
OVEREXPRESSION OF C-FABP INDUCES METASTASIS

The increased expression of metastasis-promoting genes is an important pathological step in initiating the malignant dissemination of cancer cells. In the present work, we have identified C-FABP as one of the major genes whose expression is substantially changed between benign and malignant human prostate and breast cell lines (Fig. 1). This difference in the levels of expression of C-FABP mRNA was confirmed to be 5–17-fold higher in prostate and 6.5-fold higher in breast malignant cell lines compared with the benign cell lines. Similar differences were also observed at the protein level (Table 1). Moreover, the highest levels of C-FABP were found in malignant metastasis-derived cell lines with the highest invasive abilities. Thus, there was a close correlation between high-level expression of C-FABP and malignant characteristics of the human cell lines (Table 1).

When an in situ hybridization technique was used to examine the expression of C-FABP mRNA in human prostate tissues, approximately 70% of the malignant samples were positive, approximately 10% were partially positive, and only about 20% were negative. In contrast, approximately 80% of the BPH tissue samples were negative, and only 10.6% were partially positive, and none were positive (Table 2). The results demonstrated that the number of malignant prostate tissue samples expressing C-FABP mRNA was significantly higher than the number of BPHs expressing C-FABP mRNA (Fisher’s exact test, $P < 0.01$), indicating a possible important role of C-FABP in human prostate cancer. Although more malignant tissues than benign tissues expressed C-FABP mRNA, among the carcinoma tissues, there is no significant difference in the percentage of tissues expressing C-FABP between carcinomas with lower Gleason scores (scores of 1–5) and those with higher Gleason scores (scores of 5–10). Among the 39 samples with Gleason scores of 1–5, 69.2% were classified as positive. Similarly, among the 25 carcinoma samples with higher Gleason scores (scores of 5–10), 68% were classified as positive. Thus, the expression of C-FABP did not increase with increasing metastatic potential. These results indicated that C-FABP might play a more important role in the initiation of malignant changes in the early stage than in the preservation of metastasis of prostate cancer.

To test the biological significance of enhanced levels of C-FABP, we transfected the C-FABP gene into the benign rat model cell line Rama 37 to examine its metastatic capability. This well-characterized cell line has been used successfully for studying the genetic events involved in the invasion and metastasis of breast (25–28) and prostatic cancer (29). When injected into syngeneic rats, the pooled C-FABP-transfectants, which consisted of a mixture of individual cell clones with different metastatic potentials, induced metastases in a significant number of animals (Fisher’s exact test, $P < 0.02$; Table 3). Among the remaining 26 animals inoculated with pSV-C-FABP-R37 cells, 6 rats (23.1%) developed metastases. In comparison, the control pSV-R37 cells, which were produced by the vector alone, did not produce any metastases in all 30 inoculated animals. When the sublines Met-1 and Met-2, which were derived from lymph node and lung metastases, respectively, and express high levels of C-FABP, were reinoculated into the syngeneic rats, they induced metastases in an
even higher percentage (50%) of animals (7 of 14 animals; Table 3) than that observed in the first round of transfection with the pSV-C-FABP-R37 cells (23%; Fisher’s exact test, P < 0.03). The higher metastatic rate observed in the second round of the in vivo assay indicated that Met-1 and Met-2 were highly metastatic colonies selected from the transfectant pool during the first round of inoculation. The expression of C-FABP detected in the transfectant cell pool and in their sublines, Met-1 and Met-2, correlated with the ability of the cells to metastasize in vivo. The primary tumors produced by the pSV-C-FABP-R37 transfectants and all six metastases were immunocytochemically stainable by antibodies to human C-FABP (Fig. 7, b–d), whereas the tumors produced by the control transfectant pSV-R37 were not stainable under the same conditions (Fig. 7a). These results showed that the expression of C-FABP was the cause of metastasis and that the metastatic capability was transferred to the Rama 37 cells by the expression of C-FABP in a genetically dominated manner.

Analysis of the levels of C-FABP expression in rat cells showed that although there was a qualitative correlation between the presence of C-FABP and the ability of the cells to metastasize in vivo in our rat model system, there was no simple quantitative relationship between the levels of C-FABP and the frequency of metastases (Table 3). These results suggested that an additional gene(s) other than C-FABP may also be involved in generating the higher metastatic frequencies obtained with cell lines derived from the metastatic lesions. To investigate which possible genes would be involved in metastasis, we have performed some pilot experiments and demonstrated that the VEGF gene is moderately expressed in the transfectant pSV-C-FABP-R37 cells but is greatly increased in Met-1 and Met-2 cells in vitro and in all metastases in vivo. In contrast, its expression in Rama 37 cells and the control vector alone transfectant cells is barely detectable. In addition, we also found that the microvesSEL densities of the metastases and their primary tumors developed from the C-FABP expression transfectants were much higher than those in the primary tumors developed from the control vector alone transfectants. Because VEGF is a potent angiogenesis factor and can facilitate the malignant dissemination of the primary tumor cells (30), we suggest that the metastases induced by C-FABP may have been caused, in part, by up-regulating the expression of the VEGF gene. It may be possible that under a certain expression level, the increase in VEGF is closely associated with the increasing level of C-FABP. However, once the threshold level is exceeded, a further increase in C-FABP may have little additional effect on the expression of VEGF. This may explain why the simple linear relation between metastatic ability and the level of C-FABP is not found in the rat cells, whose C-FABP expression may be in excess of the threshold level.

Although the possibility that the C-FABP gene had integrated into a particular site and knocked out the function of a metastasis suppressor gene cannot be ruled out, it is more likely that the metastasis-promoting capability of C-FABP is related to its fatty acid-binding activity. C-FABP is a member of the fatty acid-binding protein family, and, like other fatty acid-binding proteins, it may play important roles in the storage and transport of fatty acids (31, 32). High levels of C-FABP, along with several other proteins, have also been detected previously in squamous cell carcinomas from the bladder (33) and other sites (24). C-FABP and its fatty acid-binding activity have been detected in endothelial cells of the microvasculature of such organs as the placenta, heart, skeletal muscle, small intestine, lung, and renal medulla as well as in Clara cells and goblet cells of the colon (34). In addition, adipocyte FABP, another member of the FABP family, was increased in transitional cell carcinomas (35, 36), indicating a possible role in initiating malignant transformation of the bladder cells.

Because fatty acids have recently been identified as signaling molecules (37), which can be recognized by a nuclear receptor, PPAR (38), the elevated expression of FABP may give rise to an increased total uptake of fatty acids and hence an enhanced fatty acid signaling activity. Moreover, previous studies have shown that excessive levels of free fatty acids may be translocated into the nucleus to activate a target gene through PPAR-γ and may contribute to colon carcinogenesis (39–41). Recent evidence indicated that this target gene might be VEGF because it was demonstrated that VEGF production was stimulated by PPAR-γ agonists (42). More recently, it has been demonstrated that the expression of caveolin is greatly increased in prostate and breast cancer (43). Caveolin is a major protein constituent of caveolae, a recognized subcompartment of the plasma membrane and Golgi network. Thus, the highly elevated expression of caveolin in prostate and breast cancer may indicate the increased number of caveolae. Because the fatty acid transport protein CD36 is localized in the caveolae, the increased number of caveolae along with the caveolin may indicate an increased amount of synthesis of CD36, a further indication of increased fatty acid transporting and perhaps signaling activity in prostate and breast cancer.

From the results reported in this work and those reported previously, we suggest that there may be a novel fatty acid signaling pathway in prostate and breast cancer, and it is through this possible pathway that the elevated expression of C-FABP induces the malignant dissemination of the DNA recipient cells in our Rama 37 model.

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OVEREXPRESSION OF C-FABP INDUCES METASTASIS


Identification of the Messenger RNA for Human Cutaneous Fatty Acid-binding Protein as a Metastasis Inducer

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