Dysregulation of Annexin I Protein Expression in High-Grade Prostatic Intraepithelial Neoplasia and Prostate Cancer

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INTRODUCTION

The completion of the Human Genome Project, along with vast improvements in gene and protein expression analyses, will provide researchers an unprecedented opportunity to study and understand molecular events responsible for human cancer development. Two major challenges for successful cancer investigators will be the extraction of useful molecular information from relevant tissue samples and efficient analysis of vast genomic and proteomic data sets. These challenges are being addressed by the development of molecular profiling techniques aimed at discovering differential gene and protein expression associated with cancer development and progression. The recent development of LCM facilitates the precise procurement of a pure population of cells from a histology section and has improved the acquisition of biologically relevant molecular information from human tissue samples (1).

Although CaP is the most common noncutaneous malignancy among men in the United States; the critical molecular events underlying its development remain poorly understood. In the past, tissue heterogeneity and the lack of useful initiation models have made molecular studies of CaP particularly challenging. Combining LCM with gene and protein expression studies has shown promise in overcoming these barriers (2–6). Gene and protein expression has been successfully analyzed by cDNA microarrays and two-dimensional gel electrophoresis, respectively, from LCM-procured prostate epithelium (3–6). When differential gene and protein expression patterns in CaP cells were compared with patient-matched BPE, several dysregulated (either over- or under-expressed) genes and proteins were found (2–5). One gene found to be down-regulated in CaP at both the mRNA and protein level was annexin I (lipocortin I; Ref. 2).

Annexin I is one of 11 members of a recently described family of calcium-dependent binding proteins. The gene-encoding annexin I maps to chromosomal band 9q and was initially cloned as a phospholipase A2 inhibitor (7). In addition to regulating arachidonic acid production, annexin I may be a critical mediator of apoptosis (8–9). Other functions attributed to annexin I include regulation of hepatocyte growth factor receptor signaling (10), facilitation of epidermal growth factor receptor degradation, and control of intracellular calcium release (11). To date, there are limited data on annexin I expression levels in human cancers. Annexin I overexpression has been observed in gastric and breast...
cancer, but the biological consequence of these findings remains unclear (12). Loss or dramatic reduction of annexin I protein expression in 20 cases of squamous cell carcinoma of the esophagus and 25 cases of CaP has been reported recently (2). These findings are the first evidence suggesting that reduced expression of annexin I protein may be associated with the development of human cancer.

In this investigation, we demonstrate that annexin I protein expression is reduced in HGPIN and androgen-dependent CaP compared with BPE using IHC. Q-PCR demonstrated that annexin I mRNA was reduced in CaP compared with BPE, suggesting that dysregulation of annexin I in CaP occurs at the level of transcription.

MATERIALS AND METHODS

Annexin I expression protein and mRNA levels were studied in a cohort of men with clinically localized prostate cancer. Protein levels were assessed by IHC scored visually and measured by mage analysis, and mRNA levels were measured by Q-PCR.

IHC

Specimens. Formalin-fixed, paraffin-embedded tissue sections from 69 radical prostatectomy specimens obtained from men with clinically localized CaP between 1994 and 1998 were studied. All sections selected for study contained both benign and malignant glands. Forty-five cases also contained HGPIN. The mean patient age was 61.3 ± 7 years. Forty-one percent of the patients were African Americans and 59% were Caucasian Americans. The mean preoperative PSA was 12.6 ± 10.7 ng/ml, and the mean Gleason sum was 6.7 ± 1.0. Fifty-four percent of cases were pathologically organ-confined, and 6% demonstrated lymph node metastasis.

IHC. Six-millimeter histological sections were the avidin-biotin IHC technique (13) was applied by capillary action and modified for the MicroProbe staining station (Fisher Scientific, Pittsburgh, PA; Ref. 14). In brief, after deparaffinization and rehydration, tissue sections were heated to 100°C for 30 min in a steamer in the presence of antigen-retrieval solution [Citra (pH 6.0); Biogenex, San Ramon, CA] and cooled for 5 min. Slides were preincubated with 2% normal horse serum for 5 min at 37°C and washed with automation buffer. Mouse IgG anti-annexin I monoclonal antibody (Transduction Labs, Lexington, KY) diluted 1:100 was used as the primary antibody and incubated overnight at 4°C. Biotinylated mouse IgG [diluted 1:200 in PBS (pH 7.4)] was used as the secondary antibody for 15 min at 37°C followed by avidin-biotin complex (Vector Labs, Inc., Burlingame, CA) for 15 min at 37°C. We visualized the antibody-antigen complexes using 0.75 mg/ml 3′,3′-diaminobenzidine (Aldrich Chemical Co., Inc., Milwaukee, WI) with nickel in distilled water for 8 min at 37°C. Slides were dehydrated through graded alcohol solutions, cleaned with Hemo-De xylene substitute (Fisher Scientific), mounted with Permount (Fisher Scientific), and cover slipped.

Visual Analysis. For each case, a genitourinary pathologist (S. J. M.) examined one adjacently stained histological section stained with H&E to identify CaP and HGPIN lesions. HGPIN was stringently identified with the criteria established by Mc-Neal and Bostwick (15) and Bostwick et al. (16). Staining intensities of BPE, HGPIN, and CaP were graded on a scale of 0 to 3+ (0 = no staining, + = trace staining, 3+ = maximal staining) in an annexin I-immunostained adjacent section. Fifteen randomly selected histological slides were previewed to help establish a reference point for our visual scores. Patient characteristics including age, race, preoperative PSA, Gleason grade, and pathological stage were obtained from a clinical database and compared with immunostaining intensity.

Digital Image Analysis. The imaging hardware consisted of a Zeiss LSM 200 microscope in bright-field mode, an Optronics camera (Optronics, Goleta, CA), a camera control unit (Optronics), and a personal computer (Apple Inc, Cupertino, CA). Reference images were acquired randomly within encircled areas containing BPE, HGPIN, or CaP. Intensity histograms were generated with NIH image software (Scion, Frederick, MD) to avoid under- or oversaturation of gray levels, and the gain and the brightness of the camera control unit were adjusted to avoid under- or oversaturation of gray levels. Images exhibiting one of the three pathological entities of interest (BPE, HGPIN, or CaP) were sampled randomly throughout each histological section with a grid system to prevent biased acquisition of images. Each field of view was digitized at a total magnification of ×600 with a ×20 objective (numerical aperture = 0.6).

Image areas corresponding to cellular areas stained by DAB with nickel enhancement or hematoxylin were segmented from red, green, and blue parts of a color image with an adaptive local thresholding method (17). Three binary images were added by a logical odds ratio operation to obtain a better segmentation of cellular areas. To minimize the impact of artifact, necrosis, empty lumens, and other areas that were not of interest (i.e., stroma or heavy lymphocytic infiltrate), Optimas image analysis software (Optimas Corporation, Bothell, WA) was used to isolate areas of interest and set threshold criteria such that only epithelial glands containing BPE, HGPIN, or CaP were analyzed. We measured the mean optical density of each immunopositive cell using Optimas to calculate a logarithmic ratio of the mean gray value of the immunopositive area and the mean gray value of the background. For each image, mean optical density representing each cell type was obtained by calculating the mean of all absorbance values measured from immunopositive areas (18–19). The mean and SE of the mean optical density for each cell type (HGPIN, CaP, BPE) for each patient was obtained from 11 sampled images. Eleven images of each cell type (BPE, HGPIN, and CaP) for each histological slide (total images per slide = 3 × 11 = 33) provided an adequate sample size because the relative error of the mean optical density became <10% with a 95% confidence interval. Overall mean optical density values for each interval cell type (a mean of all absorbance values for that cell type) were calculated for all patients. The impact of patient clinical and pathological characteristics on MOD was determined.

Statistical Analysis. Visual scores and mean optical density values were analyzed with Statgraphics statistics software (STSC, Inc., Rockville, MD) and Wilcoxon. P < 0.05 was considered significant.
Real-Time Fluorescence Q-PCR

Specimens. Of the 65 patients studied by routine IHC, 14 cases were selected for Q-PCR RNA analysis; these cases had matched specimens of prostate tissue in a tissue bank. Each selected case contained one section with a minimum of 80% malignant cells as determined by H&E staining and another section from the peripheral zone opposite the side of the CaP containing BPE. High-quality RNA was assessed by modification of the techniques of radical prostatectomy. The vascular pedicles were left intact until the entire specimen was ready for excision. All radical prostatectomy specimens were inked and incised, and specimens acquired were snap frozen in the operating room by the urological surgeon (J. L. M.).

Clinical data from all patients, including age, race, PSA, tumor stage, and Gleason grade, were acquired from a clinical pathological database. A single genitourinary pathologist (S. J. M.) reviewed histology and assigned Gleason grades for all tissue specimens.

Real-Time Fluorescence Q-PCR. The ABI PRISM 7700 (Perkin-Elmer/Applied Biosystems, Foster, CA), a 96-well thermal cycler, permits real-time activation and peak fluorescence measurement via an array of optical fibers coupled to a laser, spectrophotometer, and a charge-coupled device. A gene-specific and nonextendible oligonucleotide probe was designed and labeled with a reporter fluorescent dye (FAM) at the 5' end, and a quencher dye, 6-carboxy-tetramethylrhodamine, at the 3' end (20). The probe was hybridized to the target cDNA between the 5' and 3' oligonucleotides, and as amplification proceeds, the 5'-fluorophor is cleaved off the probe by 5'-nuclease activity of the polymerase. Free in solution, the 5'-fluorophor is no longer quenched by FAM, leading to an increase in fluorescence at 518 nm.

Fluorescence intensity produced during the PCR amplifications in each well was monitored every 8.5 s. A real-time amplification plot was generated for each well, where the number of amplification cycles were plotted on the X axis and the log of change in fluorescence over baseline (\(\Delta Rn = \text{fluorescence} - \text{baseline fluorescence}\)) was plotted on the Y axis. The software of the instrument was used to calculate a threshold cycle number (\(Ct\)) at which each PCR amplification reached a significant threshold level. This threshold cycle was directly proportional to the initial number of specific mRNA template copies present in the sample.

Annexin I-specific mRNA Assay. We obtained the cDNA sequence for annexin I was from GenBank using the Genetics Computer Group analysis programs (Wisconsin Package Version 10.1; Genetics Computer Group, Madison, WI). An annexin I-specific 5' oligonucleotide (taagggggcggcggcga), a 3' oligonucleotide (ttatgccgagcgcagcatc), and an intervening fluorescent dye-labeled probe (tgagcccctatcctaccttcaatccatc) were fashioned with Primer Express (Perkin-Elmer/Applied Biosystems). The probe was synthesized, labeled with 3'-6-carboxy-tetramethylrhodamine and 5'-FAM, and high-performance liquid chromatography purified (Integrated DNA Technologies, Coralville, IA). Final concentrations of oligonucleotides were 400 nM, and probes were at 200 nM. Amplification parameters were 30 s at 48°C and 10 s at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

Annexin I synthetic mRNA (cRNA) was used as the positive control and absolute standard. In brief, full-length annexin I cloned in the pTZ19R phagemid bearing the T7 promoter was obtained from ATCC (ATCC 65114; Manassas, VA). This DNA construct was linearized with DRAIII and used to in vitro transcribe annexin I cRNA with the MEGAscript kit (Ambion, Austin, TX). Amplifications of 2-fold serial dilutions of the annexin I cRNA were used to construct standard curves and determine the dynamic range of our assay. With this absolute standard, we could accurately measure the annexin I message over the 5-log range from 0.1 fg to 2 \(\times 10^5\) fg.

Inaccuracies of RNA quantitation and pipetting inefficiency introduce well-to-well and sample-to-sample RNA loading differences. Ribo-green (Molecular Probes, Eugene, OR) fluorescence was used to accurately quantify starting levels of total RNA extracted from the tumor samples. To reduce further inaccuracies in measuring specific message one-step RT-PCR approach was used. By integrating RT and PCR amplification in one tube, no error was introduced by comparing cDNA synthesized under different conditions, and pipetting steps were reduced.

RNA Isolation. Total RNA was isolated with a guanidinium isothiocyanate-based system (RNeasy; Qiagen, Valencia, CA). Snap-frozen tumor specimens were power homogenized (Powergen; Fischer Scientific, Pittsburgh, PA) before chloroform extraction. RNA contamination prevented by treatment with 3 units of DNase (RNase free; Ambion, Austin, TX) and 40 units RNAsin (Promega, Madison, WI) for 20 min at 37°C. Further control for genomic DNA contamination was secured by the inclusion of reactions lacking the reverse transcriptase (RT-PCR) for each of our unknowns.

RNA samples from all matched prostate tumors and BPE were tested on the same day with the same master reaction mixture to obviate experimental variability. Total RNA (10 ng) was assayed per well and each tissue sample was tested in triplicate. A mean annexin I mRNA expression level in femto-grams was then calculated for CaP and matched BPE samples.

Statistical Analysis. The mean and SD for the three mRNA measurements were calculated and comparisons between patient-matched cancer and benign samples were made with a paired t test, and \(P < 0.05\) was considered significant.

RESULTS

The vast majority of BPE glands (67/69, 97%) were scored by visual assessment as either “2+” or “3+.” In contrast, BPE, the majority of CaP (61/69, 88%), and HGPIN (41/45, 91%) were scored at “1+” or lower. There were no cases in which CaP or HGPIN was scored as “3+. The distribution of visual scores for BPE, HGPIN, and CaP are presented in Table 1. An example of an IHC section with the corresponding H&E section (Fig. 1, A and B) demonstrates the differences in immunohistochemical staining among BPE, HGPIN, and CaP.

Analysis of visual scores comparing benign and malignant glands within the same specimen revealed a consistent trend of decreased staining in HGPIN and CaP compared with BPE. BPE consistently stained more intensely than either HGPIN (40/45, 91%) or CaP (65/69, 93%). Overall, the mean visual scores for both HGPIN (0.76 ± 0.61) and CaP (0.83 ± 0.56) were signifi-
Significantly lower \( (P < 0.05) \) than the mean score for BPE \( (2.2 \pm 0.41) \). In 67% \( (46/69) \) of CaP and 76% \( (34/45) \) of HGPIN cases, reduction in staining exceeded one visual score level. No cases revealed staining of HGPIN or CaP to be greater than the BPE within the same specimen. Levels of annexin I protein expression did not correlate with Gleason grade, pathological stage, or race.

Digital image analysis is summarized in Table 2 and revealed an overall mean optical density of 0.34 for BPE \( (n = 759; 69 \text{ patient cases } \times 11 \text{ images}) \), an overall mean optical density of 0.14 for CaP \( (n = 759; 69 \text{ cases } \times 11 \text{ images}) \), and an overall mean optical density of 0.16 for HGPIN \( (n = 495; 45 \text{ cases } \times 11 \text{ images}) \). Overall mean optical density values for both HGPIN and CaP when compared with BPE were significantly different \( (P < 0.05 \text{ and } P < 0.05, \text{ respectively}) \), whereas the overall mean optical density of HGPIN compared with CaP was not \( (P = 0.08) \). The mean optical densities for the individual cases are presented in Fig. 2, which plots the mean optical density of the three pathological elements of interest (BPE, HGPIN, and CaP) as a ratio to the mean optical density of benign glands. As was seen with visual scoring, there were no cases where, within the same specimen, the mean optical density of HGPIN or CaP was greater than the mean optical density for BPE. In 97.7% \( (44/45) \) of HGPIN and 98.5% \( (68/69) \) of CaP, staining intensity was significantly reduced \( (P < 0.05) \) when compared with BPE in the same specimen. Mean absorbance values for HGPIN and CaP within the same specimen were usually similar and significantly different \( (P < 0.05) \) in only 15.6% \( (7/45) \) of cases. Mean absorbance values did not correlate with Gleason grade, pathological stage, or race.

Results of Q-PCR were consistent with the immunohistochemical analysis (Fig. 3). Use of an absolute standard (annexin I cRNA) in each assay allowed calculation of absolute femtograms/10 ng of total RNA extracted from each sample. The CaP tissue specimens expressed a mean of 762 ± 362 (range, 199–266) annexin I copies/10 ng of total tumor RNA. The matched specimens of BPE expressed a mean of 1295 ± 418 (range, 346–2051) annexin I copies/10 ng of total RNA. Of the 14 matched sets, 10 cases \( (72\%) \) exhibited significantly lower mRNA expression in CaP compared with BPE \( (P < 0.05) \). In 5/14 \( (36\%) \) annexin I mRNA expression was reduced by >50%.

### Table 1

Results of visual scoring of immunostained sections

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Score</th>
</tr>
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<tbody>
<tr>
<td>BPE ( (n = 69) )</td>
<td>0</td>
</tr>
<tr>
<td>HGPIN ( (n = 45) )</td>
<td>7 (15.6%)</td>
</tr>
<tr>
<td>CaP ( (n = 69) )</td>
<td>8 (11.6%)</td>
</tr>
</tbody>
</table>

Table 2 Results of digital image analysis

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Mean</th>
<th>±SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPE</td>
<td>0.342</td>
<td>0.7785</td>
<td>0.201–0.560</td>
</tr>
<tr>
<td>HGPIN ^a,b</td>
<td>0.157</td>
<td>0.0467</td>
<td>0.085–0.264</td>
</tr>
<tr>
<td>CaP ^c,d</td>
<td>0.139</td>
<td>0.0389</td>
<td>0.055–0.224</td>
</tr>
</tbody>
</table>

\^a HGPIN vs. BPE \( (P = 0.00) \).
\^b HGPIN vs. CaP \( (P = 0.08) \).
\^c CaP vs. BPE \( (P = 0.00) \).
\^d CaP vs. HGPIN \( (P = 0.08) \).
DISCUSSION

Recent molecular-profiling studies have identified annexin I dysregulation as a potentially important event in prostate cancer development (2). Our current study supports this initial hypothesis by demonstrating that significant reduction in annexin I protein expression occurs in the vast majority of HGPIN lesions (97.7%) and CaP (97.7%). The possibility of subjective biases has been eliminated by determining annexin I staining intensity with image analysis as well as with subjective visual assessment by an experienced genitourinary pathologist. Because findings from visual scoring and digital image analysis were the same, our conclusion that annexin I protein expression is reduced in HGPIN and CaP appears valid.

Although the stepwise progression from HGPIN to invasive CaP has not been proven conclusively, clinical (21) and transgenic (22) studies strongly support HGPIN as a precursor to CaP. Genetic events shared by both HGPIN and CaP are known. Loss of heterozygosity at chromosomal bands 8p, 10q, and 16q have been reported in both HGPIN and CaP (23), suggesting that a tumor suppressor gene may be located in these regions. Reduced expression of glutathione S-transferase pi (GSTP1) as a result of hypermethylation has been described in HGPIN and CaP (24). Given these associations, molecular events relevant to the formation of HGPIN are also likely to be important in CaP initiation and development.

Because HGPIN and CaP lesions exhibit similar levels of
Dysregulation of Annexin I Protein Expression

Annexin I protein expression, it is likely that reduction in annexin protein expression is an important early event in CaP initiation. Our observation that reduced annexin I protein expression is closely associated with histological changes of HGPIN further supports this notion. Even within the same gland, BPE maintains immunopositive annexin I staining, but individual cells exhibiting phenotypic changes characteristic of HGPIN show markedly reduced staining (Fig. 1, B and C).

Although our study cannot determine whether loss of annexin I expression causes or is associated with HGPIN, studies of annexin I function suggest possible tumor suppressor functions. Annexin I was initially cloned as the phospholipase A2 inhibitor and is an important mediator and down-regulator of arachidonic acid production (7–8). In light of the growing evidence regarding the impact of cyclooxygenase (COX)-2 inhibition on CaP prevention and treatment (25, 26), the role of annexin I in prostaglandin synthesis may prove to be an important contributory and explanatory factor. Studies have shown that decreased annexin I expression in macrophages leads to prolonged activation of extracellular signal-related kinase (27). Increased extracellular signal-related kinase activation has been observed in CaP (28) and could be responsible for enhanced cellular proliferation. Annexin I also plays a role in apoptosis, regulation of hepatocyte growth factor receptor signaling, and degradation of epidermal growth factor receptor. Alterations in these pathways have been implicated in CaP development.

The mechanism for annexin I dysregulation in HGPIN and CaP is not understood. Because cDNA array experiments (3) and Q-PCR demonstrate reduced annexin I mRNA in CaP compared with BPE, annexin I dysregulation probably occurs at the genomic or transcriptional level. This study demonstrated reduced annexin I protein expression in 98.5% of cases, but reduced mRNA in 72% of cases; it is possible that more than one mechanism could be responsible for annexin I protein dysregulation. An alternative explanation for this discrepancy exists because LCM was not used to procure cells for mRNA analysis and the four tissue samples not showing difference in mRNA levels may have been contaminated with BPE, endothelium, or inflammatory cells.

Decreased annexin I protein and mRNA expression in HGPIN and CaP compared with BPE suggest an alteration at the level of gene transcription or mRNA stability. Genomic deletions or mutations of the promoter and coding region could lead to decreased mRNA transcription and reduced mRNA stability, respectively. Chromosomal abnormalities are commonly associated with HGPIN and CaP. Loss of heterozygosity has been demonstrated most often on chromosomal bands 8p, 10, 13 and 16 (29–32), but it is possible small deletions or bp mutations may have been missed on chromosomal band 9q (29–32). Chromosomal gains have been detected for 9q in prostate cancer (32).

Because annexin I expression is reduced but not completely eliminated, it is likely that dysregulation occurs at the level gene transcription. Promoter hypermethylation is a defect commonly observed in prostate cancer and is responsible for reduced expression of several important regulatory genes (33–39). However, because annexin I does not contain CpG islands in the promoter region or coding sequence, methylation is unlikely to be responsible for reduced annexin I expression observed in CaP. Therefore, a more likely explanation for the mechanisms for annexin I dysregulation involves the alteration of one or more proteins that regulates annexin I transcription.

This report is important for two reasons: (a) it demonstrates that dysregulation of annexin I expression may be an important early event in prostate carcinogenesis; and (b) it confirmed that a protein alteration found by exploratory molecular-profiling studies is a common finding in clinical specimens. Completion of the human genome sequence, improved microscopic tissue procurement (i.e., LCM), and more refined gene and protein expression analysis will allow us to enhance our molecular and histopathological understanding of CaP and continue to identify new gene and protein alterations important in human cancer development and progression. Ultimately, there will be new opportunities to more accurately assess CaP risk, design effective prevention strategies, improve therapeutic options, and individualize treatment.

Conclusions. These data exhibit a consistent and markedly reduced expression of annexin I protein in HGPIN and CaP cells compared with BPE in the same radical prostatectomy specimen. The consistent finding of down-regulation in both HGPIN and CaP suggests that annexin I dysregulation is an important early event in CaP development. Reduced annexin I mRNA expression confirmed the immunohistochemical findings and suggested that dysregulation occurs at the transcriptional level. The fact that these results were the end product of a molecular profiling approach supports a valuable role for this experimental method for the identification of biologically important changes in human cancer.

REFERENCES
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