Loss of Annexin II Heavy and Light Chains in Prostate Cancer and Its Precursors

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Abstract

Annexin II mRNA coding for a calcium binding protein was found to be absent in prostate cancer by subtractive hybridization and Northern analysis. In contrast to high expression in normal and benign hyperplastic glandular and basal epithelium, Annexin II heavy (p36) and light (p11) chains in 31/31 prostate cancer specimens were lost immunohistochemically. In glands involved by prostate intraepithelial neoplasia, 65% lost both chains in glandular epithelial cells, whereas basal cells were all positively stained. Southern analysis of cancer DNA showed no noticeable deletion in p36 gene. LNCaP cells treated with 5-aza-cytidine re-expressed p36, suggesting methylation could be responsible for the silencing.

Introduction

Identification of genetic alterations is a necessity to gain insight into neoplasia and can provide new tools for diagnosis, treatment, and prevention. Prostate cancer is one of the most frequently occurring carcinomas in men and has become the second most common cause of cancer-related death. However, our understanding of its etiology and the multistep progression is very limited. Oncogenes and tumor suppressor genes known to be associated with other malignancies have a remarkably low frequency of mutation or deletion in prostate cancer. The most common change is the loss of Glutathione S-Transferase [GST] p36, suggesting methylation could be responsible for the silencing.

Materials and Methods

Prostate Specimens. The entire prostate gland from radical prostatectomy patients was taken to pathologists immediately after surgical removal. Biopsies were made from the posterior prostate surface (4). Fresh normal prostate tissue was obtained from two sources. One was a biopsy specimen where no cancer cells were found. The other was a brain-dead organ donor. The peripheral zone of the prostate from the organ donors was dissected from the transitional/central zone using urethra and ejaculatory ducts as the landmarks. Specimens used for immunohistochemistry were fixed in 10% formaldehyde solution and paraffin embedded. Benign hyperplastic tissue was obtained from transurethral resection. Informed consent was obtained from the patients or families, and the study protocol was approved by the Central Sydney Area Health Service Ethics Review Committee.

RNA/DNA Extraction. Before nucleic acid extraction, fresh cancer specimens and normal prostate were microsectored (5). The tissue was homogenized in TRI-reagent (Sigma Chemical Co., Sydney, NSW, Australia). Total RNA and DNA were isolated following the manufacturer’s protocol with the following modification regarding DNA. After precipitation of DNA, the pellet was washed 3 times with 75% ethanol to remove residual phenol. The pellet was then digested overnight at 55°C in a lysis buffer [50 mm Tris, 100 mm EDTA, 0.5% SDS, and 0.3 mg/ml proteinase K (pH 8)]. DNA was isolated by standard phenol/chloroform:isoamyl alcohol technique.

Suppressive Subtractive Hybridization. Poly(AD)- RNA was isolated from total RNA of a cancer specimen and a normal prostate of an organ donor using Dynabeads oligo(dT)25 (DYNAL, Oslo, Norway). Using PCR-Select cDNA subtraction kit (Clontech, Palo Alto, CA), subtracted libraries were subcloned into pGEM T-easy vector (Promega, Madison, WI). Individual recombinant colonies were randomly picked and inoculated into 96-well plates containing l-broth and ampicillin. Cloudy medium was used as template for PCR amplification of the insert sequence. PCR products were dot blotted onto membranes. Duplicate membranes were hybridized to subtracted radiolabeled cancer and normal cDNA library, respectively. The sequence of differentially expressed clones was determined by single direction sequencing.

RT-PCR. Total RNA (2 µg each) was reverse transcribed into cDNA. For p36, PCR was performed using primers derived from the nucleotides 971–991 and 1213–1229 (GenBank accession no. D000017). For p11, PCR was performed using primers derived from the nucleotides 138–158 and 376–394 (GenBank accession no. M38591). HPRT was amplified as a loading control (5′-TTACCTTTCTACACACCGTGTA-3′ and 5′-TTGCTGACCTGCTGT-GATTACATCA-3′). Thermocycling included an initial denaturation at 94°C for 2 min; 24 cycles (p36 and p11) and 30 cycles (HPRT) of denaturation (94°C for 30 s), annealing (54°C for 30 s), and elongation (68°C for 30 s). The amplified DNA was separated on a 1% agarose gel.

Northern and Southern Analyses. Total RNA (15 µg) was denatured for 15 min at 65°C and electrophoresed using a 1% formaldehyde agarose gel. The RNA was transferred onto Hybond N nylon membrane. A PCR product (nucleotides 971–1229) of p36 cDNA was used as a probe. The membrane was hybridized in ExpressHyb solution (Clontech) containing 1 × 106 cpm of denatured probe overnight at 68°C. Genomic DNA (10 µg each) was digested using HindIII, PstI, and BglII. After overnight digestion at 37°C, DNA was electrophoresed in a 0.8% agarose gel and transferred onto nylon membranes after depurination, denaturation, and neutralization. Radiolabeled probes were prepared from two overlapping PCR products covering the entire open reading frame of p36 gene. Hybridization conditions were identical as described above.

Immunohistochemistry. Tissue sections (5 µm) were incubated for 1 h at 37°C after microwave antigen retrieval with a mouse monoclonal anti-p36 antibody (Zymed Laboratories) diluted 1/1000 and 1/200; 1/400 in 1% preimmune rabbit serum. Omission of the primary antibody from the sample was used as the negative control. The signal was amplified using the avidin-biotin-peroxidase complex system (Vectors Laboratories, Burlingame, CA) and visualized using the liquid 3,3′-diaminobenzidine substrate-chromogen system (Dako, Carpenteria, CA). Isotype and method controls were performed for each sample by substituting the primary antibody with preimmune mouse IgG (Dako) and 1% preimmune mouse serum, respectively. For p11, a mouse monoclonal anti-p11 antibody (Transduction Laboratories) diluted 1/1000 was used. For 34BE12 and PSA, the antibodies were diluted 1/100 (Dako) and 1/600 (Dako), respectively. Dako Envision + peroxidase (mouse

Received 4/27/01; accepted 7/11/01.

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1 The abbreviations used are: PIN, prostate intraepithelial neoplasia; RT-PCR, reverse transcription-PCR; HPRT, hypoxanthine phosphoribosyltransferase; DAB, 3,3′-diaminobenzidine; PSA, prostate-specific antigen; BPI, benign prostatic hyperplasia.

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K4001 for 34βE12 and rabbit K4003 for PSA) was used as the signal detection system.

5-Azacytidine Treatment. LNCaP cells (0.5 × 10⁶) were cultured in T-medium (6) with 10% heat-inactivated fetal calf serum (FCS) for 2 days. A final concentration of 5 μm of 5-azacytidine was added to the culture medium from a freshly prepared 5 m stock solution in DMSO. After 5 days the cells were harvested and RNA isolated using TRI-reagent. The RNA was DNase treated, and RT-PCR was performed as described above using p36 primers.

Results

Identification of Loss of Annexin II (p36) mRNA in Prostate Cancer. Using suppressive subtractive hybridization between two microselection-derived prostate mRNAs, one from a previously untreated 64-year-old prostate cancer patient and the other from a 25-year-old brain-dead, cancer-free organ donor, we identified a cDNA sequence that was absent in prostate cancer. Sequencing revealed a full match with Annexin II heavy chain (p36) from nucleotide position 657 to 1357 (GenBank accession no. D00017). RT-PCR and Northern analysis using total RNA from additional six normal prostate and seven cancer specimens confirmed that expression of p36 was significantly reduced in prostate cancer (Fig. 1, A and B).

Annexin II (p36) Protein in Normal Prostate, BPH, Prostate Cancer, and PIN. We analyzed p36 immunohistochemically in normal and diseased prostate (Fig. 2). In peripheral (n = 10) and transitional/central zones (n = 6) of normal prostates, the apical and lateral (i.e., circumferential) plasma membrane of glandular and ductal epithelial cells showed high expression of p36. Similarly, the basal cells also expressed p36 protein. In BPH tissue derived from transurethral resection of prostate (n = 12), p36 was expressed in the same cell type as normal tissue with comparable intensity. In contrast, a loss of p36 expression in cancer cells was observed in 31/31 randomly selected individual cancer blocks with a Gleason score range of 3–9 (5.7 ± 1.4, mean ± SD), whereas adjacent normal and hyperplastic glands in the same tissue block had positive expression. The proportion of cancer cells in each block that had no p36 expression was nearly 100% in 28 cases and close to 50–75% in the other 3 cases. The Gleason score of the 3 cases was 5, 6, and 5, respectively. One of them was the cancer case 5 in the Northern analysis (Fig. 1B). Cytokeratin 34βE12, a marker of prostate basal cells (7), was used to verify that the p36-positive cells were indeed cancer cells, because they lacked a participating basal cell component.

We additionally examined the p36 expression in high-grade PIN. Twenty-one of the 31 cancer blocks had foci of high-grade PIN at the interface between cancer and non-neoplastic glands. Each block had 6 or 7 glands involved by high-grade PIN, making the total 130 glands examined. There was a clear difference in p36 expression in this morphologically indistinguishable, high-grade PIN. Of them, 65% showed negative and 35% showed positive, apical staining whereas all of the basal cells were positively stained. Persistent expression of 34βE12 in p36-negative PIN ruled out the possibility of cancer (Fig. 2). PSA-positive staining confirmed that p36-negative PIN was prostate gland origin (data not shown).
Annexin II (p11) Protein in Normal Prostate, BPH, Prostate Cancer, and PIN. Because Annexin II light chain (p11) is always in complex with p36, the absence of p36 expression in prostate cancer and in 65% of PIN prompted us to examine p11 expression immunohistochemically (Fig. 2). In normal and BPH, p11 was expressed in the same cell type as p36. Prostate cancer cells had also lost p11 expression. The small fraction of p36-positive cancer cells in three cases also expressed p11. However, we noticed in a few cancer cells that weak p11 expression was present, whereas p36 was lost. p36-negative or -positive PIN remained negative or positive for p11. Interestingly, despite the absence of p11 protein in prostate cancer, p11 mRNA levels remained unchanged when compared with normal prostate (Fig. 1C).

Association of Hypermethylation with Silencing of Annexin II (p36) Gene Expression. To address the question of whether the decrease in p36 mRNA level is a result of a homozygous deletion in p36 coding regions in cancer DNA, Southern analysis with three different endonucleases was conducted using p36 open reading frame sequence as the probe on genomic DNA from paired cancer tissue and blood leukocyte of the same patients (n = 8). We found no difference between cancer and blood DNA (data not shown). To establish if the p36 gene has a CpG island spanning the promoter region and could therefore be susceptible to hypermethylation, we used the full-length p36 cDNA sequence to search for p36 genomic DNA sequences from a high-throughput genomic DNA database and obtained a sequence from clone AC019146. Analysis of the upstream region of the gene has shown a CpG island spanning the promoter, first exon, and intron of the gene (Fig. 3). To determine whether methylation is associated with Annexin II silencing in prostate cancer, we treated prostate cancer LNCaP cells, which do not express p36, with 5-azacytidine (Fig. 4). p36 is expressed in normal prostate, blood, and DU145 prostate cancer cells but is silent in prostate cancer and LNCaP cells. Annexin II expression was reactivated in LNCaP cells after 5-azacytidine treatment suggesting that methylation is associated with Annexin II silencing in prostate cancer.

Discussion

Annexin II can be found in vivo as p36, p36p11, and p36p112, and is involved in endocytosis, exocytosis, and membrane trafficking (8). p36 belongs to a family of calcium- and lipid-binding proteins and is a substrate for receptor and nonreceptor protein kinases (9, 10). Recently, membrane p36 has been shown to mediate steroid rapid action (11). p11 is a small calcium binding protein and shares sequence homologies with the S-100 family (12). Overexpression of p36 increases p11 protein level with no change in its mRNA levels (13), suggesting that p36 has a post-translational stabilizing effect on p11 protein. Thus, it is possible that the lack of p36 in prostate cancer gives rise to substantial degradation of p11 so that the rate of translation from p11 mRNA fails to compensate. The observation that negative p36 expression coexists with a weak expression of p11 in a small number of cancer cells may represent the early stage of p11 degradation. As the loss of p36 and subsequently p11 in prostate cancer obligates all forms and functions of Annexin II, it is difficult to know which function of Annexin II is related to prostate carcinogenesis. However, the importance of calcium, the key element for Annexin II to function, is well appreciated in programmed cell death in the prostate (14, 15). The distinctive expression pattern of Annexin II among BPH, PIN, and cancer indicates that the loss of Annexin II is specific for prostate cancer. The loss of expression in 31/31 individual prostate cancer blocks regardless of the Gleason score and 65% of high-grade PIN suggests that Annexin II is involved in an early stage or common pathway of prostate carcinogenesis. Recently, Annexin I has been found to be absent in prostate cancer and PIN by Western blot and immunohistochemistry (16), and Annexin VII can suppress proliferation of prostate cancer cells and is significantly reduced in metastatic and recurrent prostate cancer (17). Thus, Annexin could represent a previously unrecognized mechanism involved in prostate cancer. The clinical implication of residual Annexin II-positive cancer cells in three cases will be closely followed up.

To address the question of whether the >50% decrease in p36 mRNA level is a result of a homozygous deletion in the p36 coding region in cancer DNA, we conducted Southern analysis on genomic DNA from paired cancer tissue and blood leukocyte. Because our prostate cancer cases are sporadic, a somatic homozygous deletion in the p36 coding region in the cancer DNA should be revealed by comparing it with the paired blood DNA. Any point mutations or small deletions within the coding region of the p36 gene, which Southern analysis may not be able to detect, should not cause such a substantial decrease in p36 mRNA levels. We found no difference thus far between cancer and blood DNA, suggesting that the decrease in p36 mRNA level is likely attributable to alterations affecting the expression or processing of mRNA. Because DNA hypermethylation is commonly associated with silencing of tumor-related genes (1–3), a preliminary study was conducted to examine the possible association of methylation with Annexin II silencing in prostate cancer. The finding that the p36 gene does have a CpG island in the promoter region and in the first exon and, more importantly, that p36 expression could be reactivated by treatment of prostate cancer LNCaP cells with a demethylation agent indicates that hypermethylation could be responsible for silencing of the p36 gene in vivo. However, because 5-azacytidine can also affect Sp1 transcriptional activity (18), additional study by bisulphite sequencing of normal and cancerous prostate-derived DNA is needed to verify and identify methylation sites that distinguish cancer cells from normal cells. Although p36-positive cancer cells of case 5 was demonstrated by both Northern analysis and immunohistochemistry, it remains to be seen whether additional mechanisms besides mRNA transcription are involved in the down-regulation of the p36 protein, considering the fact that 28 of 31 cases had no p36 protein, whereas one of five cases showed unchanged p36 mRNA.

This study also provides evidence that high-grade PIN is not biochemically homogenous. It is known that not all PIN will progress to cancer (19); however, the lack of a progression-associated “marker” has prevented pathologists from stratifying the morphologically un-
distinguishable PIN. Although additional studies are required to correlate the p36-negative and -positive PIN with the incidence of cancer and prognosis, the fact that the vast majority of cancer cells show negative expression for p36 suggests that the p36-negative PIN is the true precursor of cancer. We speculate that p36-positive PIN cannot progress to cancer without loss of p36 expression first. However, because we have encountered three cases containing a small fraction of p36-positive cancer cells, it remains a possibility that p36-positive PIN can develop directly to p36-positive cancer.

A differential diagnosis among well-differentiated cancer, PIN, and non-neoplastic lesion is often required for needle-core biopsy, in particular when the number of glands is insufficient and/or the quality of a specimen is unsatisfactory (20). Currently, 34βE12 is the “gold standard” by highlighting the absence of basal cells in infiltrating cancer. However, it is problematic to additionally differentiate PIN from non-neoplastic glands because basal cells are present in both. The unique expression pattern of Annexin II-negative PIN, absent in glandular epithelial but present in basal cell, is in contrast to benign hyperplasia in which Annexin II is expressed in both cells and to the vast majority of cancer cells in which Annexin II expression is abolished and basal cells are no longer attached. Thus, identification of Annexin II provides a potential diagnostic marker for needle-core biopsy specimens.

References

Announcements

MEETING OF THE RADIATION RESEARCH SOCIETY

The annual meeting of the Radiation Research Society will be held at the State University of Iowa, Iowa City, on June 22–24, 1953. The Society will be the guest of the University, and all meetings will be held on the campus. The program will consist of: (1) Two symposia, one on "The Effects of Radiation on Aqueous Solutions," which includes the following speakers: E. S. G. Barron, Edwin J. Hart, Warren Garrison, J. L. Magee, and A. O. Allen. The second is "Physical Measurements for Radiobiology" and companion talks by Ugo Fano, Burton J. Moyer, G. Failla, L. D. Marinelli, and Payne S. Harris. (2) On Monday night, June 22, a lecture by Dr. L. W. Alvarez on meson physics has been tentatively scheduled. On Tuesday night, June 23, Dr. L. H. Gray of the Hammersmith Hospital, London, will speak on a topic to be announced. Dr. Gray's lecture is sponsored by the Iowa Branch of the American Cancer Society. Those desiring to report original research in radiation effects, or interested in attending or desiring additional information, please contact the Secretary of the Society, Dr. A. Edelmann, Biology Department, Brookhaven National Laboratory, Upton, L.I., New York.

ERRATUM

The following correction should be made in the article by Beck and Valentine, "The Aerobic Carbohydrate Metabolism of Leukocytes in Health and Leukemia. I. Glycolysis and Respiration," November, 1952, page 891; substitute for the last paragraph:

The data in Table 5 permit several interesting calculations. If one compares the amount of glucose actually disappearing with the sum of the amount equivalent to lactic acid produced plus that equivalent to $O_2$ consumption, it is seen that the amount of glucose "cleavage products" exceeds the amount of glucose utilized by 12 per cent in N and 27 per cent in CML and is exceeded by the glucose utilized by 16 per cent in CLL. If the assumption is made that, in this respect, the myeloid and lymphoid cells of leukemia are similar to those of normal blood, it may be that the computed normal figure represents a summation of the myeloid (M) and lymphoid (L) cells that make up the normal leukocyte population. Thus, if $M = +0.27$ and $L = -0.16$ and the normal differential is 65 per cent M and 35 per cent L, then

$$0.65 (+0.27) + 0.35 (-0.16) = +0.12,$$

a figure identical to the observed $+0.12$ for normal leukocytes.
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Cancer Res 2001;61:6331-6334.

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