Differential Expression and Androgen Regulation of the Human Selenium-binding Protein Gene hSP56 in Prostate Cancer Cells

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ABSTRACT

Low levels of dietary selenium are associated with increased risk of malignancy of several organs, including the prostate. Using a subtractive approach called linker capture subtraction, we have found that the human selenium-binding protein gene hSP56 is differentially expressed by the relatively slow-growing, androgen-sensitive prostate cancer cell line LNCaP but not by the more rapidly growing androgen-insensitive lines PC-3 and DU145. We confirmed this differential expression by Northern blot analysis. Importantly, hSP56 expression by LNCaP cells was reversibly down-regulated by exogenous androgen in a concentration-dependent manner. Marked differences in steady-state hSP56 mRNA levels were found in a variety of normal and neoplastic human cells that were examined. hSP56 expression was especially high in normal tissues that appear to benefit from the cancer-protective action of dietary selenium and was low in many neoplastic cells. The results suggest that hSP56 may play a role in determining the neoplastic phenotype.

INTRODUCTION

Selenium is a trace element that is essential for a number of biological processes (1, 2). It was first associated with cancer risk more than two decades ago (3, 4). Indeed, a deficiency of dietary selenium is associated with an increased incidence of a number of malignancies, including cancers of liver, lung, colon, prostate, and pancreas (5-10). The relationship of selenium to cancer was recently underscored by a clinical trial in which supplementation of dietary selenium for cancer prevention resulted in significant reduction in total cancer mortality, total cancer incidence, and incidences of prostate, lung, and colorectal cancer (11). However, despite the strong association of selenium deficiency with malignancy, the precise mechanism of this effect is not understood.

Selenium-containing proteins are of two general classes. First, true selenium-containing proteins contain selenium in the form of selenocysteine, which is integrated into the polypeptide chain. Examples include glutathione peroxidase (12), selenoprotein P (13), and thioredoxin reductase (14). Second, there are selenium-binding proteins. Members of this heterogeneous group are identified by virtue of their ability to bind selenium. Examples of these include liver fatty acid-binding protein (15), protein disulfide isomerase (16), and SP56 (17, 18). The mode of binding of selenium to these proteins is not well characterized, and for the most part, the functional significance of the selenium binding is not known. Therefore, a relationship of these proteins to the neoplastic process remains to be established.

Recently, we developed a highly efficient method for isolating genes expressed differentially by similar cell types (19). We have begun to apply this method, designated LCS, to human prostate cancer in an effort to identify genes expressed differentially in highly aggressive forms and in more indolent varieties. We now report that hSP56 (20), the human homologue of SP56 (17, 18), is expressed by the relatively slow-growing, androgen-sensitive human prostate cancer cell line LNCaP but is not expressed by either of two more rapidly growing androgen-insensitive human lines, PC-3 and DU145. Moreover, hSP56 expression in LNCaP cells is down-regulated reversibly by androgen. Its expression levels in normal human tissues suggest a possible link to malignancies associated with selenium deficiencies.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. Human prostate cancer lines LNCaP, PC-3, and DU145 (American Type Culture Collection, Rockville, MD) and normal human B-lymphoid cell line B142 (kindly provided by Dr. DiCioccio, Roswell Park Cancer Institute, Buffalo, NY) were cultured in RPMI 1640 with 10% FBS in 95% air-5% CO2 at 37°C. In some experiments, cells were grown under the same conditions, except that 10% androgen-depleted DCS (HyClone, Logan, UT) was used instead of normal FBS. For the androgen regulation experiments, specified concentrations of DHT (Sigma Chemical Co., St. Louis, MO) were added to the medium containing 10% DCS.

RNA Isolation and cDNA Preparation. Total RNA was isolated by a guanidium thiocyanate/phenol method (21). Poly(A)+ RNA was selected through oligo(dT)25-Dynabeads (Dynal Inc., Lake Success, NY). cDNA was synthesized from poly(A)+ RNA using a SuperScript Choice System (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer’s instructions. Oligo(dT)15-18 was used to prime the first strand of cDNA synthesis.

LCS. The original method was reported by Yang and Sytkowski (19). Briefly, 2 µg of each of two mRNA populations prepared from LNCaP or PC-3 cells were converted to double-stranded cDNAs, fragmented with AluI and Rsal, and ligated to linkers that had a blunt end and a 2-base 3' protruding end: ACTCTTGCTT- GACGAGCCTCT and ACTGAGAACGACCTGCCTGAGA-3'. The cDNAs were amplified by PCR using the first strand (designated AP) as the primer. The reaction (100 µl) contained 10 mM Tris-HCl (pH 8.9), 50 mM KCl, 0.1% Triton X-100, 200 µM dNTPs, 1 µM AP, 2 mM MgCl2, 1 µM of melted agarose, and 5 units of Taq polymerase (Promega), running for 30 cycles (94°C for 1 min, 55°C for 1 min, and 72°C for 1 min). The amplified cDNA fragments were purified using a Gene-Clean kit (Bio101, Vista, CA). To prepare driver, the amplified DNA were digested with Alul and SacI intensively to remove the linker.

The digested driver DNA (2.5 µg) and nondigested tester DNA (0.1 µg) were mixed, dried, and redissolved in 4 µl of a buffer containing 15 mM N-(2-hydroxyethyl)piperazine-N'- (3-propane sulfonic acid) and 1.5 mM EDTA (pH 8.0), overlaid with mineral oil, and denatured by heating for 5 min at 100°C. One µl of 5 M NaCl was added, and the DNA was hybridized for 20 h at 67°C. After hybridization, 20 µl of pH-shift buffer B [11 mM zinc chloride, and 10 mM Na acetate (pH 5.0)] was added, and the solution was divided into five aliquots. They were incubated with 0, 0.85, 1.75, 3.5, or 7 units of mung bean nuclease (Promega), respectively, at 37°C for 30 min. To each sample was added 80 µl of pH-shift buffer B [10 mM Tris-HCl (pH 8.9), 50 mM KCl, and 0.1% Triton X-100]. They were heated (95°C for 5 min) to inactive the mung bean nuclease. Then 20 µl of enzyme solution [10 mM Tris-HCl (pH 8.9), 50 mM KCl, 0.1% Triton X-100, 1 mM dNTPs, 5 µM AP, 10 mM MgCl2, and 5 units of Taq polymerase (Promega)] was added. The PCR was run under same conditions as above. Each sample was electrophoresed on 2% agarose gel. The sample with the most abundant products of 0.1-1.0 kb was selected as tester for another round of subtraction.

After three rounds of subtraction, the PCR-amplified products were purified, digested with SacI, inserted into pGEM-7Zf+(+) (Promega), and transformed into Escherichia coli JM109 cells. White colonies were picked randomly and inoculated into LB + Amp medium in individual wells of a 96-well
plate. Two replica DNA dot-blots were prepared on GeneScreen Plus filters using 25 µL of bacterial cells per well. The replica dot-blots were processed according to Brown and Knudson (22) and probed with random-labeled driver DNAs from LNCaP and PC-3, respectively. Candidate positive colonies were boiled for 5 min in 20 µL of H₂O and centrifuged. DNA in the supernatant was amplified by PCR using universal vector primer T7 and SP6 for 20 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The PCR products were electrophoresed on 2% agarose. The desired bands were excised and purified (Gene Clean). The products were subjected to direct DNA sequencing (23) and were used as probes in Northern blot analysis.

**Northern Blot Analysis.** Five µg of total RNA were fractionated by electrophoresis on 1% agarose gel containing formaldehyde and transferred to GeneScreen Plus membrane (DuPont). Human multiple tissues and cancer cell line Northern blots 7759-1, 7760-1, and 7757-1 were purchased from Clontech (Palo Alto, CA). The inserts from isolated clones were used to prepare probes. The probes were 32P-labeled to specific activities of ~5 × 10⁹ cpm/µg using a random-prime DNA labeling kit (Life Technologies, Inc., Gaithersburg, MD). Membranes were hybridized with the probes in a solution containing formamide, dextran sulfate, and 2X prehybridization/hybridization solution (Life Technologies, Inc.) at 42°C for 24 h, washed once in 1X SSC-0.1% SDS at room temperature for 20 min, and then twice in 0.2X SSC-0.1% SDS at 55°C for 20 min. The blots were exposed to Kodak X-OMat AR film (Eastman Kodak, Rochester, NY). Quantification was carried out by laser densitometric analysis (UltraScan XL, Pharmacia LKB, Upssala, Sweden).

**Southern Blot Analysis.** Genomic DNA was extracted from cell lines LNCaP, PC-3, DU145, and B142 according to Sambrook et al. (24). DNA samples (10–15 µg) were digested with restriction enzyme EcoRI or HindIII. LNCaP, PC-3, DU-145, and B142 genomic DNA from prostate cancer cell lines LNCaP, PC-3, and DU145 according to Sambrook et al. (24). DNA was extracted by phenol/chloroform extraction, precipitated with ethanol, and redissolved in water. The DNA was digested with restriction enzymes EcoRI and HindIII (Life Technologies, Inc.) at 37°C for 2 h. The digested DNA was run on a 0.8% agarose gel and transferred to GeneScreen Plus membrane (DuPont). The filter was hybridized to a 32P-labeled probe and further processed as described above.

**RESULTS**

**Differential Expression of a Selenium-binding Protein Gene hSP56 between Androgen-sensitive (LNCaP) and Androgen-insensitive (PC-3 and DU145) Human Prostatic Carcinoma Cells.** We used LCS (19) to isolate genes expressed differentially, either by the human prostate cancer cell line LNCaP, a relatively slow growing androgen-sensitive line, or by PC-3, a more rapidly growing androgen-insensitive line. Among the several genes isolated thus far, DNA sequence analysis identified one as nt 2–230 of hSP56 (20), the human homologue of selenium-binding protein gene SP56. Our sequence revealed an additional G residue 6 nt upstream of the ATG start codon (Fig. 1). The LCS screening procedure indicated that hSP56 was expressed by LNCaP more abundantly than by PC-3 (data not shown).

**AP56 is an SP56 homologue shown in murine systems to bind metabolites of acetaminophen. It exhibits a high degree of homology to murine SP56, differing by only 24 nt within the 1419 nt coding region. Northern blot analyses report notably no difference between the SP56 and AP56 transcripts, but RT-PCR has been successful (18).** Because the human AP56 gene, if one exists, has not yet been cloned, studies of hSP56 expression either at the mRNA or protein level must be interpreted carefully. Indeed, we propose that human hSP56 transcript should be considered as SP56/AP56 until the presumed human AP56 is cloned and sequenced.

**We confirmed differential expression of hSP56 (SP56/AP56) in human prostate cancer cell lines by Northern blot analysis.** Total cellular RNA from androgen-dependent LNCaP and from androgen-independent PC-3 and DU145 cells was electrophoresed, transferred to GeneScreen Plus, and probed with 32P-labeled hSP56 cDNA (Fig. 2). A single, relatively abundant 1.9-kb transcript was detected in LNCaP cell RNA. In contrast, no transcript was detected in either PC-3 or DU145 RNA.

To determine whether the apparent lack of hSP56 expression in PC-3 and DU145 was due to deletion or obvious rearrangement of the gene, we performed a Southern blot analysis of genomic DNA from prostate cancer cell lines LNCaP, PC-3, and...
DU145 and from a normal B-lymphoid cell line B142. All four cell lines showed identical hybridizing bands of equal intensity (data not shown). Therefore, the lack of hSP56 expression does not appear to be due to any major deletion or rearrangement of the gene. However, this analysis does not exclude the presence of one or more subtle mutations that may lead to a decrease or loss of expression.

hSP56 Expression Is Down-regulated by Androgen in a Concentration-dependent and Reversible Manner. We next investigated the possibility of androgen regulation of hSP56. Replicate cultures of LNCaP cells were grown in medium containing 10% normal FBS. Northern analysis confirmed basal levels of hSP56 (SP56/AP56) mRNA. Quantification of the Northern blot results showed a profound time- and concentration-dependent down-regulation of hSP56 expression by androgen.

**DISCUSSION**

In this study, we have used a rapid and highly efficient cloning method, LCS (19), to identify genes differentially expressed in human prostate cancer cells. We have found that the human selenium-binding protein gene hSP56 is expressed in the androgen-sensitive human prostate cancer cell line LNCaP but not in the more rapidly growing androgen-insensitive lines DU145 and PC-3. Moreover, the data show that androgen is a negative regulator of hSP56 expression in prostate cancer cells.

hSP56 was designated a human selenium-binding protein by virtue of its homology with murine SP56 (20). The murine SP56 protein,
originally designated SLP56, was first isolated from mouse liver by Medina and coworkers (17), who identified it as a selenium-binding protein. They noted that the nucleotide sequence contained no TGA codon in frame and that, therefore, it is not an authentic selenoprotein containing selenocysteine in the polypeptide chain. The mode of selenium binding to this protein is unknown. Importantly, another murine protein, designated AP56, which may play a role in detoxification of acetaminophen in the liver (25, 26), differs from SP56 by only 24 nt within the coding region (18). Within the region homologous to the partial human cDNA for hSP56 that we isolated, the murine sequences for SP56 and AP56 differ at nt +37 of the coding sequence. Assuming a similar difference between human hSP56 and a putative human AP56 (which has not yet been reported), we have identified our clone as hSP56 and not the putative human AP56. However, because the methods used for northern analysis cannot distinguish between murine SP56 and AP56 transcript, it is possible that our Northern analyses here detect both transcripts as well. Thus, in our Northern analyses, we have designated the transcript as hSP56 (hSP56/AP56).

The data show profound androgen down-regulation of steady-state levels of hSP56 (SP56/AP56) transcript. Interestingly, in the mouse, SP56 and AP56 appear to be differentially regulated (18). Thus, our data may be interpreted either as showing the presence of only hSP56 transcript in these prostate cells or that both hSP56 and the putative human AP56 are down-regulated similarly by androgen treatment. There may be a special biological significance to the down-regulation of hSP56 by androgen. It has been proposed that SP56 may have a growth-inhibitory role (17). Thus, androgen down-regulation of hSP56 could cause release of prostatic cells from such growth inhibition. The resulting increase in mitotic activity could also make these cells more sensitive to agents capable of inducing neoplastic transformation. It is to be noted that, whereas the androgen-sensitive LNCaP cells express hSP56, neither androgen-insensitive lines PC-3 or DU145 do so. These other two lines grow more rapidly and metastasize more readily. That is, their phenotype is similar to prostatic cancer cells that are “androgen stimulated.” However, neither PC-3 nor DU145 express a functional androgen receptor. This phenotype, coupled with the down-regulation of hSP56 in both lines, is consistent with a state resembling androgen stimulation that does not require the androgen receptor.

Our data indicate high levels of hSP56 expression in liver, lung, colon, and prostate but extremely low levels in cancerous forms of these tissues. Interestingly, it is just these tissues that had lower rates of neoplasia in the recent clinical trial of selenium supplementation (11). This intriguing finding may signal a role for hSP56 in the anticarcinogenic effects of dietary selenium. It will be of additional interest to determine whether androgen regulates hSP56 in these other tissues, each of which has been reported to express the androgen receptor (27). Further studies will provide insights into the role of hSP56 and selenium in prostatic epithelial growth and neoplastic transformation.

REFERENCES
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